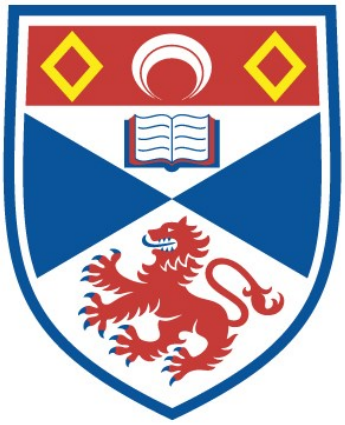
STUDIES ON NEURONAL 5-HT IN A GASTROPOD MOLLUSC, HELIX POMATIA (L.)

V. W. Pentreath

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



1973

Full metadata for this item is available in
St Andrews Research Repository
at:
http://research-repository.st-andrews.ac.uk/

Please use this identifier to cite or link to this item:
http://hdl.handle.net/10023/15025

This item is protected by original copyright

STUDIES ON NEURONAL 5-HT IN A GASTROPOD MOLLUSC, Helix pomatia (L.)

By

V. W. Pentreath

A thesis presented for the degree of Doctor of Philosophy of the University of St. Andrews.

Wellcome Laboratories of Pharmacology,
The Gatty Marine Laboratory,
The University,
St. Andrews.

May 1973

BIBLIOTHECA UNIVERSITATIS ANDREANÆ

ProQuest Number: 10170919

All rights reserved

INFORMATION TO ALL USERS
The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript
and there are missing pages, these will be noted. Also, if material had to be removed,
a note will indicate the deletion.

ProQuest 10170919

Published by ProQuest LLC (2017). Copyright of the Dissertation is held by the Author.

All rights reserved.
This work is protected against unauthorized copying under Title 17, United States Code
Microform Edition © ProQuest LLC.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

ABSTRACT

Studies on neuronal 5-hydroxytryptamine in a gastropod mollusc, Helix pomatia (L.)

V.W. Pentreath

Wellcome Laboratories of Pharmacology, Gatty Marine Laboratory,

St. Andrews University, Fife, Scotland

This thesis describes information obtained on the structure of 5-HT--containing neurons, on the mechanisms of transport of 5-HT and its precursors into and within neurons, on the nature of the blood supply to the CNS, and on the function of 5-HT-containing neurons within the CNS of Helix pomatia. In particular data is obtained for the giant serotonin-containing neuron (GSC) in each cerebral ganglion.

Dense-cored vesicles of mean diameter 100 nm are present in the perikarya and axon branches of the GSCs. Vesicles of similar appearance are present in the presumed presynaptic endings of the GSCs. Evidence is presented which suggests that such vesicles sequester 5-HT. The fine structure of presumed presynaptic endings making synaptic connections with the GSCs is described.

Following exposure to tritiated 5-HT, electron microscope autoradiography showed that silver grains, often in very high concentrations, were located over certain fine axon branches thought to be nerve endings. These processes contained small dense-cored vesicles, which were morphologically similar to those thought to sequester 5-HT in the perikarya of the GSCs. It is suggested that re-uptake into nerve endings is a mechanism of inactivation of 5-HT in the CNS of Helix pomatia.

Following exposure to tritiated 5-HTP, silver grains were observed over the perikarya of the GSCs and other known 5-HT-containing neurons. There was no indication that 5-HTP was taken up by nerve endings or by non-nervous structures.

The accumulation of tritiated tryptophan was less specific; all the neuron perikarya took up this substance.

The CNS of H. pomatia is supplied by branches of the anterior aorta. Capillaries from these branches open into a blood space which is adjacent to, and continuous over the surface of the nervous tissue. Blood passes from this space through the epineural sheath into the body cavity sinuses. Three tissue layers separate the blood spaces from the nervous tissue. These are (i) a luminal endothelium, (ii) a connective tissue layer, and (iii) glial cells. The luminal endothelium and connective tissue are freely permeable to uncharged particles of 10 nm or less.

Electrophysiological analysis showed that each GSC sends axon branches to muscles in the lips of the animal. Selective stimulation of the GSCs resulted in an increase of electrical activity recorded from these muscles, but no change in their length. This effect was mimicked by 5-HT applied to the muscles. It is suggested that the GSC has a facilitatory effect on the lip muscle potentials.

SUPERVISOR'S CERTIFICATE

I certify that V.W. Pentreath has fulfilled the conditions laid down under Ordinance General No. 12 of the University Court of St. Andrews, and is accordingly qualified to submit this thesis for the degree of Doctor of Philosophy.

DECLARATION

I declare that the work reported in this thesis is my own and has not previously been submitted for any other degree.

VITAE

I was educated at Christ's Hospital, Sussex, and the University of St. Andrews where I graduated in Zoology in 1967. The work described in this thesis was carried out between May 1969 and December 1972.

ACKNOWLEDGEMENTS

I thank Dr. G.A. Cottrell for his help during this work. Gratitude is also due to Miss M. Radley for typing the manuscript, and Mr. J. Stevenson for help with the photography.

CONTENTS

INTRODUCTION

5-Hydroxytryptamine (serotonin, 5-HT) is present in the nervous systems of representatives of all major animal phyla (for listings see Carattini and Valzelli, 1965; Erspamer, 1966; Welsh, 1968). Much work has been undertaken to elucidate its possible role as a neurotransmitter.

At present, research on 5-HT in molluscan nervous systems is in the forefront of these studies. This situation has arisen largely because it is possible to use microtechniques to study individual neurons, which is difficult in vertebrates.

The following summary covers certain aspects of 5-HT relevant to the present work. Partial and comprehensive reviews of 5-HT in molluscs and other invertebrates have recently been published (Florey, 1965, 1967; Cottrell and Laverack, 1968; Sakharov, 1970; Gerschenfeld and Stefani, 1968; Gerschenfeld, 1973).

1. Cellular localisation of 5-HT in molluscan nervous systems

In general the central ganglia of these animals are relatively rich in 5-HT (Welsh and Moorhead, 1960); typical values lie in the range 2-6 µg/g tissue for Helix (Cardot and Ripplinger, 1963; Kerkut and Cottrell, 1963) and for Aplysia (Carpenter, Breese, Schanberg and Kopin, 1972). Numerous reports (see Gerschenfeld, 1973, for listings) which have employed the Hillarp-Falck fluorescence technique (Falck, 1962), have provided evidence that some of the amine is localized within a small proportion of neuron somata in the CNS of many species. Some reports (e.g. Bardessono et al., 1972) have presented microspectrofluorimetric evidence relating the yellow fluorescence of neuron somata to the presence of 5-HT. Reserpine depletes 5-HT from molluscan ganglia (Mirolli and Welsh, 1964; Juorio and Killick, 1972a), and causes fading of 5-HT specific fluorescence from neuron somata (Dahl et al., 1966). In one situation it has been established that an identifiable neuron somata which exhibits 5-HT-specific fluorescence contains 5-HT. This neuron

occurs in the cerebral ganglia of Helix pomatia and other gastropod molluscs so far examined (see Osborne and Cottrell, 1972b). 5-HT has been detected in this neuron (termed 'giant serotonin-containing cell', 'GSC', see Cottrell, 1971a) by fluorescence histochemistry, by bioassay (Cottrell and Osborne, 1970), and by microchromatography (Osborne and Cottrell, 1972a).

5-HT-specific fluorescence has been observed in axons arising from neuron somata which themselves fluoresce specifically for 5-HT (e.g. Osborne and Cottrell, 1972b). It has also been observed in peripheral nerve trunks such as the visceral nerve of Helix (Osborne and Cottrell, 1970), and in small branches of peripheral nerves e.g. in the hearts of Aplysia (Taxi and Gautron, 1969), Venus (Loveland, 1963) and Helix (Cottrell and Osborne, 1969a).

There is less information about the localization of 5-HT within nerve endings in neuropils, although one report (Dahl et al., 1966) suggests the presence of 5-HT-specific fluorescent varicosities in neuropils in the central ganglia of Anodonta, Helix and Buccinum.

2. Subcellular localisation of 5-HT in molluscan neurons

There is no clear information on the ultrastructural localisation of 5-HT either within neuron somata or nerve endings in molluscs. In the central nervous tissue of rats (Zieher and De Robertis, 1963) and guinea pigs (Michaelson and Whittaker, 1963), 5-HT appears to be bound to vesicles which are present in synaptosomes. Cell fractionation applied to molluscan nervous tissue has provided evidence that 5-HT may be bound to unidentified particles different in density from those which bind acetylcholine (Cottrell, 1966; Cottrell and Maser, 1967), but it has also been claimed that 5-HT is bound to membranes of endoplasmic reticulum (Zs.-Nagy et al., 1965). On the other hand, the technique of Wood (1965, 1966), which allows the ultrastructural visualization of amines including 5-HT, indicates that 5-HT is sequestered

within dense-cored vesicles (mean diameter 100 nm) in the giant 5-HT-containing neuron in the cerebral ganglia of the slug Limax (Cottrell and Osborne, 1970), and in some central neurons of Anisodoris (Jourdon and Nicaise, 1970).

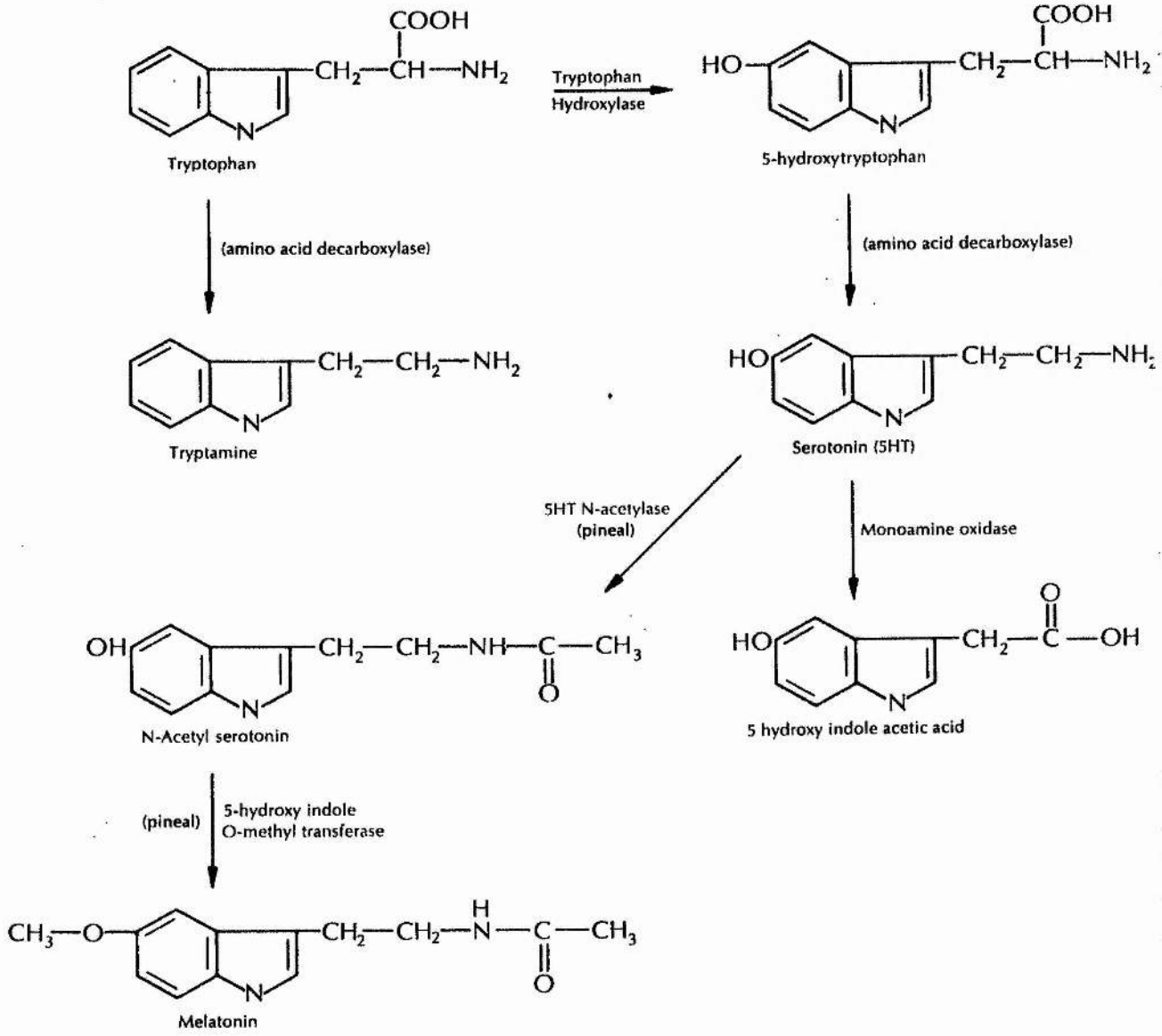
3. Synthesis of 5-HT in molluscan nervous tissue

Information on the biosynthesis of 5-HT in the nervous tissue of molluscs is not as complete as in the mammalian nervous system (Fig. 1). Although nerve tissue homogenates of several molluscan species decarboxylate 5-hydroxytryptophan (5-HTP) into 5-HT (Welsh and Moorhead, 1959; Hiripi and Salanki, 1969), and giant 5-HT-containing neurons isolated from the cerebral ganglia of Helix pomatia convert 5-HTP to 5-HT both in vitro (Cottrell and Powell, 1971) and in vivo (Osborne, 1972a), the only report on the presence of tryptophan hydroxylase, the enzyme synthesizing 5-HTP from tryptophan in the mammalian brain (see Grahame-Smith, 1971; Wurtman and Fernstrom, 1972), in molluscan nervous tissue is that by Cardot (1971a, 1972). The latter worker showed that central ganglia of Helix isolated in the blood of the animal could convert tryptophan to 5-HTP.

4. Transport and turnover of 5-HT in molluscan nervous tissue

The distribution of 5-HT-specific fluorescence within individual neurons of several lamellibranch and gastropod species (Dahl et al., 1966), indicated a high concentration of the amine within the cell body, the proximal axon, and the presumed nerve terminal, but not along most of the length of the axon. This may indicate transport from the cell body along the axon to presynaptic storage sites at the terminals (Cottrell and Laverack, 1968). Ligatured visceral nerves of Helix pomatia show accumulation of 5-HT-specific fluorescence on the side of the ligature nearest the visceral ganglia, which suggests a proximo-distal transport of 5-HT along axons within these nerves (Osborne and Cottrell, 1970).

1


Tryptophan
Tryptophan Hydroxylase
5-hydroxytryptophan
(amino acid decarboxylase)
(amino acid decarboxylase)
Tryptamine
Serotonin (5HT)
5HT N-acetylase (pineal)
Monoamine oxidase
N-Acetyl serotonin
5 hydroxy indole acetic acid
(pineal)
5-hydroxy indole O-methyl transferase
Melatonin

The rate of turnover of 5-HT in the optic ganglia of a cephalopod Eledone moschata is high (half-life no longer than 10 minutes; Bertaccini, 1961). A similar rapid turnover of 5-HT occurs in the rat brain (Neff and Tozer, 1968). There are no data on 5-HT turnover in other molluscan tissues.

5. Inactivation of 5-HT in molluscs

There are no unambiguous data on the mechanism of 5-HT inactivation either within the intraneuronal pools, or in the case of its presumed release into the synaptic cleft, in extracellular spaces.

An enzyme with monoamine oxidase (MAO) activity has been found in nervous tissue of Octopus vulgaris (Blaschko and Hawkins, 1952), and certain non-nervous molluscan tissues (see Blaschko and Hope, 1957), but the evidence for the presence of MAO in most molluscan nervous tissues is conflicting. In several species the MAO inhibitor, nialamide, has been shown to increase the number and intensity of yellow fluorescent varicosities believed to contain 5-HT (Dahl et al., 1966; Zs.-Nagy, 1967; Sakharov and Zs.-Nagy, 1968; Maredon and Kerkut, 1970; Jaeger, Jaeger and Welsh, 1971; Osborne, 1970). On the other hand, Juorio and Killick (1972b) found no quantitative increase in the concentration of 5-HT in Helix ganglia following treatment with the MAO inhibitors pargyline and tranylcypromine, whereas such drugs significantly increased the levels of 5-HT in Octopus brain. Furthermore, MAO does not appear to be capable of inactivating 5-HT in ganglia of Busycon (Mirolli, 1968), Cryptomphallus (Gerschenfeld and Stefani, 1968), or Helix (Kerkut and Cottrell, 1963; Cardot, 1963, 1964; Juorio and Killick, 1972b), although it may act on substrates such as tryptamine or tyramine (Cardot, 1966). In contrast to these findings, a recent report by Maredon (1972) indicates that central nervous tissue of Planorbis metabolizes 5-HT to 5-hydroxyindole acetic acid (5-HIAA), presumably via MAO.

In the mammalian central nervous system, the 5-HT presumed to be released from nerve endings is probably partially inactivated by re-uptake.

into the presynaptic terminals (see Iversen, 1971), or by oxidation by MAO (Blaschko and Levine, 1966) (see Fig. 1). The lack of evidence for the existence of a metabolic pathway to dispose of 5-HT in most molluscan nervous tissue has prompted proposals for other mechanisms of inactivation. These are diffusion, desensitisation (see Gerschenfeld and Steffani, 1968), or re-uptake into neurons, for example into nerve endings in the heart of Aplysia (Taxi and Coutron, 1969). Part of the present thesis attempts to show that a process of re-uptake of 5-HT into nerve endings exists in the CNS of Helix pomatia. Certain aspects of transmitter uptake are discussed in more detail in this part of the thesis (chapter 3).

In summary, there is evidence for the presence of MAO in cephalopods, but no clear data on this enzyme in the nervous tissue of other groups. Extensive work with sensitive biochemical techniques would seem necessary to confirm its presence. Furthermore it would also seem necessary to investigate the possible presence of other enzymes for the metabolism of 5-HT, such as those present in the mammalian brain (see Blaschko and Levine, 1966). In relation to this Osborne, Powell and Cottrell (1972) have suggested that 5-hydroxyindole may be a metabolic product of 5-HT in the CNS of Helix pomatia. Finally, it is possible that several mechanisms (e.g. re-uptake, diffusion, and enzymatic destruction) may exist simultaneously for the removal of 5-HT presumed to be liberated from molluscan nerve endings.

6. Fluctuations in 5-HT levels in molluscan nervous tissue

Cardot (1971b) has observed seasonal variations in the 5-HT content of ganglia and heart of Helix pomatia. The concentration was high before the onset of hibernation, decreased during it, and then rose in the months immediately following hibernation. These results have been confirmed by Juorio and Killick (1972b), who also found similar variations in the CNS of Helix aspersa.

Increases in levels of brain 5-HT during hibernation have been observed

in the hedgehog (Uusepää, 1963) and in toads (Segura, Biscardi and Apelbaum, 1967). The significance of such seasonal variations in the SE animals, and in molluscs, is not clear at present.

In the mammalian brain various authors (see Hóxy, Rouer and Glowinski, 1972 for refs.) have observed a marked circadian rhythm of 5-HT levels. This rhythm is particularly marked in the pineal, where the 5-HT concentration rises to maximum level 6 to 8 hr after the onset of light, but falls quickly after dark to a value of about one tenth that of the daily maximum (see Quay, 1965). This rhythm may result at least in part from diet-induced changes in plasma tryptophan (Fernstrom and Wurtman, 1972; Wurtman and Fernstrom, 1972; Knott and Curzon, 1972). There is no information on the presence of possible daily variations in 5-HT levels in molluscan nervous tissue.

7. Release of 5-HT by stimulation of molluscan nerves

S.-Rózsa and Perenyi (1966) detected, by chromatographic and spectrofluorimetric techniques, 5-HT in heart perfusates of Helix pomatia following stimulation of the cardio-regulator nerves. The levels of 5-HT recovered (between 5 and 10 µg for ten periods of stimulation of three minutes duration) seem suspiciously high (see Cottrell and Laverack, 1968), especially since the whole heart of Helix pomatia contains less than 1 µg of 5-HT (Cottrell and Osborne, 1969a). However, it is possible that stimulation of the cardiac nerves resulted in synthesis of 5-HT. S.-Rózsa and Perenyi (1966) also detected a second cardio-excitatory factor in the perfusate of stimulated Helix pomatia hearts, thought to be a derivative of arginine. An unidentified cardio-excitatory substance is also released from the heart of another gastropod, Strophocheilos, during stimulation (Jaeger, 1966). S.-Rózsa and Graul (1964) have shown definitively that cardio-excitation is chemically mediated. This was demonstrated with an arrangement of donor and recipient hearts similar to that devised by Loewi (1921).

Other evidence which suggests that 5-HT is released by cardio-excitatory

nerves has been obtained by the application of 5-HT antagonists, such as bromolysergic acid (BOL) and methysergide. Such drugs not only antagonize the effect of 5-HT, but also antagonize the effect of nervous stimulation in the hearts of Helix pomatia (S.-Rósza and Graul, 1964), and Mercenaria (Loveland, 1963). However, because such drugs also antagonize the effect of catecholamines on the lamellibranch heart (see p. 263 in Phillis, 1970), and interfere with nerve conduction in Aplysia (see Gerschenfeld and Stefani, 1966), these results may not be significant.

There is some evidence for the release of 5-HT following electrical stimulation in another molluscan organ. This is the anterior byssus retractor muscle (ABRM) of Mytilus edulis, which has been extensively studied by Twarog and co-workers (see below). 5-HT is thought to specifically relax the maintained contraction (catch) of this muscle (Twarog, 1967a). After prolonged repetitive stimulation of the ABRM, subsequent contractions, induced by acetylcholine or by electrical stimulation, were potentiated and relaxed more rapidly than control contractions before the repetitive stimulation (York and Twarog, 1973). Because these post-stimulation changes were closely mimicked (both in degree and time-course) by applied 5-HT, York and Twarog (1973) suggested that the initial repetitive stimulation of the muscle caused release of 5-HT from nerves innervating the muscle.

5-HT is released by electrical stimulation from the midbrain raphe (Ashkenazi, Holman and Vogt, 1972) and caudate nucleus (Holman and Vogt, 1972) of the cat. Gerschenfeld and Stefani (1968) attempted to demonstrate 5-HT release from central nervous tissue of the gastropod Cryptomphallus, by electrical stimulation of ganglia isolated from the animal. Although results indicated some release, the possibility of such release from non-nervous tissue (i.e. the connective tissue capsule) was not excluded. In relation to this, Chase et al. (1968) demonstrated electrical release of labelled 5-HT taken up previously by Aplysia ganglia, but their work was subsequently disproved because autoradiography showed that most of the labelled 5-HT was

taken up by the sheath of the ganglia (Ascher et al., 1968).

On the other hand, Osborne, Powell and Cottrell (1972) have shown that electrical stimulation (rate of 3 stimuli/sec for 1 hr) causes a slight decrease (5%) of total 5-HT in the sub-oesophageal ganglia of Helix pomatia. This was accompanied by an increase of 20% in the levels of 5--hydroxyindole, a substance which has been postulated as a metabolic product of 5-HT in the snail brain (Osborne, Powell and Cottrell, 1972). However it is not clear whether these changes are due to release of 5-HT at synapses, an increased intraneuronal turnover of 5-HT, or changes in levels of non--nervous 5-HT, for example in the ganglionic sheath, as mentioned above.

In summary, there are several pieces of evidence suggesting that electrical stimulation causes release of 5-HT from central and peripheral nervous tissue of molluscs, but in no case has this been proven.

8. 5-HT receptors on neurons of gastropod molluscs

The application of 5-HT to entire ganglia of certain gastropod molluscs results in the excitation of some neurons (Kerkut and Walker, 1961, 1962; Gerschenfeld and Tauc, 1964), and the inhibition of other neurons (Gerschenfeld and Tauc, 1961; Glaizner, 1967).

An important field of research by Gerschenfeld and co-workers (see Gerschenfeld, 1973, for references) has contributed significant data about the neuronal 5-HT receptors responsible for such effects. By virtue of the size and identifiability of some neurons in the central ganglia of such gastropods as Aplysia, Helix and Cryptomphallus, these workers have been able to apply iontophoretically 5-HT to localised areas of a particular neuron, and thus avoid the possibility of effects mediated via surrounding neurons. Various blocking drugs were employed in these experiments. To date, three different receptor types have been described; these have been named A, B, and C receptors (Gerschenfeld, 1971). A comprehensive review of this work has been given by Gerschenfeld (1973); consequently, only a brief

summary is given here.

A-5-HT receptors are present on the axon hillock and axon(s) of CILDA neurons [orthodromic stimulation of such neurons results in a long--lasting wave of hyperpolarization first described by Tauc (1959) as 'inhibition of long duration', and later referred to as 'Cells with Inhibition of Long Duration' by Gerschenfeld and Tauc (1964)] . Such receptors respond with a relatively long-lasting depolarisation, which is probably due to a change in membrane permeability to Na^+ (see Gerschenfeld, 1971). B-5-HT receptors are present on the axon(s) of some identified neurons in the snail CNS at relatively greater distances from the cell bodies than A-receptors. Their activation brings about hyperpolarization and inhibition of such neurons; this change in potential appears to be due to a change in K^+ permeability (Gerschenfeld, 1971). C-5-HT receptors have been found on only a small number of identifiable neurons in the snail CNS. These receptors also bring about inhibition of the neuron, which is due to a Cl^- influx caused by an increase of neural Cl^- permeability (Gerschenfeld, 1973). There is some evidence that A-5-HT receptors may be responsible for the synaptic activation of those neurons endowed with them, because orthodromic activation of such neurons evokes slow excitatory synaptic potentials (EPSP's) which can be blocked by the same drugs which inactivate the A-receptors (Gerschenfeld and Stefani, 1968).

These data have established that 5-HT in the central ganglia of some molluscs fulfills a fundamental property of transmitter substances, namely the ability to alter selectively neuronal membrane permeability to specific ions.

9. 5-HT and natural synaptic inputs onto gastropod neurons

There is some indirect evidence that A-5-HT receptors are involved in synaptic activation of the CILDA neurons endowed with them (Gerschenfeld and Stefani, 1968). Electrical stimulation of nerves making afferent

connections with such cells results in two different sorts of EPSP. These are 'fast' (duration 200-300 m sec) and 'slow' (duration more than 800 m sec) EPSP's (Gerschenfeld and Stefani, 1968). The 'slow' EPSP could be blocked by the same drugs (i.e. LSD 25, tryptamine and 5-HT itself) in the same concentrations which blocked 5-HT receptors, whereas the 'fast' EPSP was blocked only by hexamethonium bromide (Gerschenfeld and Stefani, 1968). In relation to these results, Gerschenfeld, Ascher and Tauc (1967) had previously shown that some neurons in Aplysia may receive excitatory inputs mediated by two different transmitters; acetylcholine and some other non-cholinergic transmitter. There is no evidence about the significance of the B and C-5-HT receptors.

Perhaps the best available evidence of a synaptic transmitter role for 5-HT in molluscs has been obtained by Cottrell (1970a, 1971a), who has identified synaptic links between the 'giant serotonin-containing cell' (GSC), and other identifiable neurons in the CNS of Helix pomatia. Various data on the GSC, obtained by Cottrell and co-workers, such as its structure (Cottrell and Osborne, 1970), metabolism (Cottrell and Powell, 1971; Osborne, 1972a), and amine content (Osborne and Cottrell, 1972a) have been described above. Recent work has shown that the GSC of Helix pomatia, which is situated in the metacerebral ganglion, sends out axonal branches to the buccal ganglia (Cottrell, 1970a). Selective stimulation of the GSC results in low amplitude EPSP's in some large neurons in the buccal ganglia (Cottrell, 1970a, 1971a). The buccal neurons are depolarised by 5-HT, and both reserpine and the 5-HT antagonist 'Methysergide' block the EPSP whereas LSD-25 in low concentrations potentiates it (Cottrell, 1970a, b). Imipramine, a potent inhibitor of the uptake of 5-HT by blood platelets (De Prada and Pletscher, 1968), and which blocks the uptake of 5-HT by the intact brain of Helix pomatia, also markedly potentiates the EPSP's (Cottrell, 1971b, c). Extra evidence showing that the EPSP's in the buccal neuron(s) result from monosynaptic excitation from the GSC has been obtained by injecting the GSC with TEA [tetraethyl ammonium

chloride: This substance interferes with K^+ permeability and by this means prolongs the duration of action potentials and hence increases the amounts of transmitter released (see Hille, 1970)]. After this procedure both the action potentials of the GSC and the resulting EPSP's in the buccal cells were considerably prolonged (G.A. Cottrell, personal communication).

These data suggest very strongly that some of the 5-HT present in the GSC's of Helix pomatia serves a transmitter role via a monosynaptic excitatory link with other neurons in the buccal ganglia. However it should be noted that the EPSP's recorded in the buccal neurons have very small amplitude, and that relatively high frequencies of GSC discharge are necessary to cause the buccal neurons to fire spikes. It would therefore seem necessary to have other examples of links between identifiable 5-HT-containing and follower neurons before accepting these findings as conclusive evidence that 5-HT has a synaptic transmitter role in the snail CNS.

10. 5-HT and 'catch' relaxation in a molluscan smooth muscle

The anterior byssus retractor muscle (ABRM) of the mussel, Mytilus edulis, has been used by Twarog and co-workers as a model for the study of neuromuscular transmission in polecypod molluscs. When this muscle is caused to contract by acetylcholine or electrical stimulation, tension is maintained after stimulation has ceased. The maintained tension (catch) is not dependent on continuous nervous activity, and does not require a continuation of the active state of the contractile mechanism (Twarog, 1954). Contraction is probably controlled by excitatory nerves which release acetylcholine, while relaxing nerves, which may release 5-HT, bring about the relaxation of catch (Twarog, 1967a).

5-HT specifically relaxes catch tension in the ABRM (Twarog, 1967a, b, 1968). This relaxation is not accompanied by changes in membrane potential (Twarog, 1960), but 5-HT was observed to affect markedly the electrical response of muscle cells to excitor nerve stimulation. It lowered the threshold

to nerve stimulation, increased the amplitude and frequency of spike potentials, and enhanced recovery of the preparation (Hidaka, Ooa and Twarog, 1967; Twarog, 1968; Twarog and Hidaka, 1971). However, 5-HT does not appear to change the Cl^-, Na^+ or K^+ permeabilities of the muscle (Twarog, Cottrell and Munooka, 1971), and it has been suggested that 5-HT causes an increase in the Ca^{++} fluxes from the muscle (Twarog, 1968; Minihan and Davies, 1965). Recently Bloomquist and Curtis (1972) have confirmed this suggestion. These workers studied ^{45}Ca efflux from resting ABRM, and found that at least three compartments contained the exchangeable calcium in the ABRM. Each compartment had a different time course of exchange. The most rapidly exchanging compartment (time course approx. 10 min) was thought to represent extracellular space. A second compartment (time constant approx. 80 min) was suggested as representing a membrane Ca^{++} store, and a third compartment (time course approx. 10 times that of the second compartment) was thought to represent sarcoplasmic reticulum and contractile protein. 5-HT increased the ^{45}Ca efflux from the third compartment (Bloomquist and Curtis, 1972), thus supporting a previous suggestion (Curtis and Bloomquist, 1971) that 5-HT causes Ca^{++} efflux from a slowly exchanging compartment.

The mechanism of this proposed intracellular action of 5-HT is not yet clear. 5-HT has no direct action on the isolated contractile system of the ABRM (Ruegg, 1963). It has been postulated that intracellular free Ca^{++} may decrease because 5-HT causes binding of this cation (see Twarog, 1968). In relation to this there is evidence that 5-HT may diffuse into smooth muscle cells (e.g. Weiss and Rosecrans, 1971). Alternatively, Bloomquist and Curtis (1972) consider that at least a part of the ^{45}Ca efflux from the ABRM obtained with 5-HT application, is the result of an interaction of 5-HT with receptors at or near the muscle cell surface. There is limited evidence implicating cyclic AMP as a messenger in the triggering of relaxation, after an action of 5-HT at the muscle membrane (Cole and Twarog, 1972, see below).

These data suggest strongly that 5-HT could be responsible for relaxing

catch in the ABRM (see also Gerschenfeld, 1973). Other evidence is the presence of 5-HT in the pedal ganglion, which innervates the ABRM, and in the ABRM itself (Twarog, 1954), and the release of 5-HT from the ABRM following electrical stimulation (York and Twarog, 1973; see above). However it should perhaps be noted that the ABRM is a very specialized type of muscle, and the role of 5-HT in this situation may therefore also be highly specialized. On the other hand, Wilson and Larimer (1968) have presented evidence which suggests that catch is a part of the spectrum of responses available to many muscles, and that muscles like the ABRM are unusual only in the extent to which they are specialized for catch. Little is known about the role of 5-HT in other molluscan smooth muscles, except that it causes relaxation when applied exogenously (e.g. the muscles of the gastropod radula apparatus; Hill, 1958; Fänge and Mattison, 1958).

11. Involvement of 5-HT with nucleotides in molluscs

Many cells contain an internal hormone-like messenger molecule, namely adenosine 3', 5'-monophosphate (cyclic AMP), the formation of which is triggered when agents such as catecholamines, 5-HT, or glucagon react with receptor sites on such cells (Monsour, 1966; Robison, Butcher and Sutherland, 1968). Cyclic AMP is produced from adenosine triphosphate (ATP), by the enzyme adenyl cyclase (Sutherland, Rall and Menon, 1962). The control of certain biochemical processes in vertebrates via cyclic AMP, which is the 'second messenger' concept developed by Sutherland and co-workers (see Sutherland and Robison, 1966), is now relatively well established.

Several pieces of evidence have recently implicated an action of 5-HT on cyclic AMP in molluscs. Cottrell and Osborne (1969a) have shown that adenyl cyclase is present in the heart of Spisula solida, and that the enzyme is stimulated with 5-HT. On the other hand these workers could obtain no consistent effects by applying cyclic-AMP directly to the heart, although they could not rule out permeability barriers preventing movement of cyclic AMP into the cells of the heart. Nevertheless, Cottrell and Osborne (1969a) concluded

that cyclic AMP was involved in some way in the response of the heart to 5-HT. S.-Rózsa and Pécsi (1968) have shown that several groups of nucleotides (adenine, guanine, uracil and cytosine) influence the beating of isolated hearts of Helix pomatia and Anodonta cygnea. The significance of these results in relation to 5-HT is not yet clear, however. These nucleotides did not have any influence on the effect of 5-HT on the hearts (S.-Rózsa and Pécsi, 1968).

Cole and Twarog (1972) tested the effects of cyclic AMP, and drugs which act on the adenyl cyclase system, on the ABRM of Mytilus. Of the adenine nucleotides tested, only dibutyryl cyclic AMP caused relaxation. The relaxing effect of 5-HT on the muscle was potentiated by theophylline, an inhibitor of phosphodiesterase (this enzyme breaks down cyclic AMP), but decreased by nicotinic acid, which is thought to stimulate phosphodiesterase activity and thus decrease cyclic AMP levels (Krishna, Weiss, Davies and Hynie, 1966). Although there were several inconsistencies in their results (Cole and Twarog, 1972), and these workers did not make direct measurements of cyclic AMP levels, they provide some evidence that cyclic AMP may mediate relaxation of catch.

There are however data which suggests that 5-HT stimulates the transformation of inactive phosphorylase to active phosphorylase (this name applies to a group of numerous enzymes which catalyse the transference of glycosylic groups, and which are activated by cyclic AMP) in the heart of Lymnaea stagnalis (S.-Rózsa, 1969a). 5-HT stimulated glycogen synthesis in this tissue, and it was concluded that this result was consistent with the second messenger concept, according to which transmittor substances may exert their effect by regulating different enzyme systems (S.-Rózsa, 1969a).

More direct evidence on the effect of 5-HT on cyclic AMP has been obtained by Cedar and Schwartz (1972). 5-HT, and to a slightly lesser extent dopamine, markedly stimulated formation of cyclic AMP both in isolated ganglia, connectives and identified nerve cell bodies of Aplysia californica. This

stimulation also took place in isotonic sucrose in the absence of external ions, indicating that the action of 5-HT on cyclic AMP synthesis was independent of transmitter release resulting from synaptic potentials (Cedar and Schwartz, 1972). Because the action of 5-HT on cyclic AMP synthesis was widely distributed in the nervous system, it was suggested that receptors for the response were localised throughout the nervous system. In relation to this Cedar, Kandel and Schwartz (1972) have shown that electrical stimulation of nerves and connectives of Aplysia also caused stimulation of adenyl cyclase, but to a lesser extent than that caused by the exogenous application of 5-HT to ganglia described above. Cedar and Schwartz (1972) suggested that the effect of electrical stimulation might be mediated via release of serotonin or dopamine restricted specifically to synaptic areas, whereas 5-HT in the bathing solution might affect both synaptic and non-synaptic receptors which are distributed throughout the nervous system, as adenyl cyclase appeared to be. The work of Gerschenfeld and Stefani (1968) (see also Gerschenfeld, 1973), which showed that cell bodies of Aplysia, and other molluscan neurons, most of which are free of synapses, have receptors to 5-HT along their entire surfaces, is consistent with this suggestion.

In the vertebrate CNS, the greatest increases in cyclic AMP are evoked by noradrenaline and histamine, whereas 5-HT and dopamine produce only small or insignificant effects (Krishna et al., 1970). Cedar and Schwartz (1972), on the other hand, found little or no effect on adenyl cyclase by these substances in Aplysia. The reason for these differences is not clear at present.

Although nothing is yet known about the role of cyclic AMP in the function of molluscan neurons, hearts or muscles, nor the location or nature of receptors by which 5-HT may effect this role, these data provide interesting evidence for a function of 5-HT other than that of controlling conductance changes and ionic mechanisms at membranes. Furthermore, these data are also interesting in relation to the hypothesis of Quay (1968), that 5-HT may have ubiquitous

'significant chemical interactions with specific nucleotides.' It may, perhaps, be also relevant that some transmitter substances, including 5-HT, are stored bound to ATP in mammals (see Roberts, 1966), although again there is no information that this situation exists in molluscs.

12. 5-HT and molluscan hearts

The involvement of 5-HT in nervously-induced cardio-acceleration of molluscs is well known. However the precise role it plays is far from clear. Most of the available data concerns hearts of some gastropods (especially Helix, Aplysia and Limnea) and lamellibranchs (especially Mercenaria). Certain aspects of 5-HT in molluscan cardiac tissue (i.e. cyclic AMP, seasonal variation, and release) have been summarised above.

Kerkut and Cottrell (1963) found that the heart of Helix aspersa contains 3 µg 5-HT/g fresh tissue. It appears that the amine is not distributed evenly between both chambers of the heart. There is about twice the concentration of 5-HT in the auricle as in the ventricle in the hearts of Helix (Cottrell and Osborne, 1969a), and of Aplysia (Carpenter et al., 1971). Amine-specific fluorescent structures (method of Falck, 1962), which are presumed to be axon varicosities, are present in the hearts of Helix (Osborne, 1970; Cottrell and Osborne, 1969a; Cardot, 1971c), Limnaea (Cardot, 1971c), Limax (Osborne and Cottrell, 1971) and Aplysia (Taxi and Gautron, 1969). On the other hand S.-Mónsa and Sa.-Nagy (1967), using the same technique, have previously suggested that 5-HT is present in heart muscles of Limnaea. This result has not been repeated. There is furthermore some disagreement on the distribution of the presumed 5-HT-containing axons within the heart. Cottrell and Osborne (1969a) found that their distribution in Helix pomatia paralleled a 'sparse' distribution of nerve fibres observed with methylene blue staining. Cardot (1971c) on the other hand, found that in the same species, amine-specific-fluorescent structures were markedly concentrated at the auriculo-ventricular junction. Furthermore Osborne and Cottrell (1971) reported that the

auricle of Limax maximus contained a greater number of fluorescent fibres than the ventricle.

An added complication is the specificity of the observed fluorescence. Because the fluorescent structures appeared yellow-green in colour, several of the above authors (Cardot, 1971a; Osborne and Cottrell, 1971; Cottrell and Osborne, 1969a) suggested that there was a close association of primary catecholamines with 5-HT in the hearts of Helix and Limax. Some support for this idea has been obtained by the modification of the colour of the observed fluorescence after the application of blocking drugs and precursors (see Cottrell and Osborne, 1969a). On the other hand S.-Rôzsa and Zs.-Nagy (1967) reported the presence of catecholamine-specific fluorescent cell bodies in the auricle of Limnea. These workers postulated a stepwise release of possible transmitter substances as follows: natural excitatory transmitter from cardiac nerve (unknown substance) ⟶ catecholamines from nerve cell bodies in heart ⟶ 5-HT activated within the heart cells showing 5-HT specific fluorescence (see above) ⟶ contractile process. Much evidence will be necessary to confirm this suggestion.

Cottrell and Osborne (1969a) have shown that the method of Wood (1965, 1966) stains some granules of mean diameter 115 nm in the heart of Helix. It is possible that some of these granules sequester 5-HT.

More work is necessary to clarify the localisation of 5-HT in molluscan hearts. The use of microspectrofluorimetry may resolve the specificity of the observed fluorescence. However some of the inconsistencies may possibly be explained in terms of the several neurosecretory substances of unknown nature which are thought to be present in the snail heart. These may have fluorescent characteristics similar to those of catecholamines and/or 5-HT, and may be present in the same or closely associated structures. The morphology of such proposed neurosecretory elements has been described in Helix by Cottrell and Osborne (1969a, b) and Cardot and Herold (1971), and in Limnaea by Cardot and Herold (1971) and Zs.-Nagy and S.-Rôzsa (1970) (see

also S.-Rózsa and Zs.-Nagy (1967) for a discussion of possible neuro-endocrine regulation in the snail heart.)

5-HTP is present in the heart of Helix pomatia (Osborne, Brial and Neuhoff, 1971), and its application increases yellow fluorescence in nerves within the heart (Cottrell and Osborne, 1969a). Cardiac tissue of Helix can convert 5-HTP to 5-HT (Welsh and Moorhead, 1959), and para-chlorophenyl-alanine, which is an inhibitor of tryptophan hydroxylase in the mammalian brain, markedly depletes 5-HT in the same tissue (Cardot, 1971a). However it is not known whether peripheral axons in the molluscan heart synthesize 5-HT locally *in vivo*, or whether 5-HT is supplied to such axons via axoplasmic transport from cell bodies, which may lie in the central ganglia (i.e. visceral, parietal, and cerebral ganglia; see Gubisza and S.-Rózsa, 1969; MacKay and Gelperin, 1972). In relation to this Osborne and Cottrell (1970) have shown that there is a flow of amines, including 5-HT, along the visceral nerve (which supplies the heart) of Helix pomatia.

Enzymes capable of inactivating 5-HT do not appear to be present in molluscan hearts (Cardot, 1964). Alternatively, Carpenter et al. (1971) have shown the auricle of Aplysia can accumulate almost ten times the quantity of 5-HT available in saline surrounding the heart. The uptake by the auricle is depressed when Na^+ is removed from the saline, and/or ouabain or desmethylimipramine was added to the bath saline. These results suggest that the accumulation of 5-HT in the heart of Aplysia is an active process, which may occur in nerve terminals (Carpenter et al., 1971). In relation to this Taxi and Gautron (1969) have shown by autoradiography that axons in the heart of Aplysia accumulate labelled 5-HT (see also section on inactivation of 5-HT in molluscs). Alternatively, diffusion away from the heart may be a mechanism of its inactivation.

Other evidence implicating a role of 5-HT in the control of molluscan hearts has been obtained from pharmacological studies. For example, excitation of the Mercenaria heart following nerve stimulation is blocked with 5-HT

antagonists (Loveland, 1963), and immersion of the Mercenaria heart in reserpine results in the loss of excitatory response to nerve stimulation (Loveland, 1963; see also reviews by Hill and Welsh, 1966, and Welsh, 1971). Recent pharmacological studies on the heart of Limax maximus have been described by MacKay and Gelperin (1972), and on Helix pomatia by S.-Rózsa (1969b).

In general the data summarized above suggests that 5-HT is involved in cardio-acceleration in molluscs. However, the mechanism by which this is effected is not clear. The possible implications with cyclic AMP and other enzymes have been discussed above. It may perhaps be concluded that the excitatory effect is not performed simply at a neuromuscular level, but may result from a chain-like sequence of several processes. Several as yet unidentified cardio-excitatory substances are known to be present in molluscs (e.g. Substance X in Mercenaria ganglia, Cottrell, 1966; a substance released from the heart of Strophocheilos during stimulation, Jaeger, 1966; and a substance present in tissue extracts of Helix aspersa, Korkut and Laverack, 1960). 5-HT may have an effect indirectly via, or in association with, such substances. Furthermore, 5-HT may have effects on many systems (e.g. enzymatic, neuroendocrine, ionic) simultaneously within the molluscan heart. Such systems may also vary with external factors such as season and temperature.

13. Outline of the present thesis

In the previous pages an attempt has been made to abstract data on certain aspects of 5-HT relevant to its possible neurotransmitter function in molluscs.

The work described in the present thesis was undertaken in an attempt to extend knowledge in several of thosefields summarized above. In particular, the CSO's of Helix pomatia have been employed under the assumption that they may serve as a model system for studying neuronal 5-HT. The work is described in the following order:

1. The anatomy of the GSC in Helix pomatia: An attempt is made to describe in detail the gross morphology, the anatomical connections and fine structure of the GSC soma and its axon branches, with reference to the localisation of 5-HT. An attempt is also made to identify synapses onto the GSC, and presynaptic endings of the GSC (see 3).

2. The blood supply to the CNS of Helix pomatia: A description is given of the arterial systems to the central ganglia of the animal. Electron microscopic tracers are employed in an attempt to elucidate the possible presence of barriers between blood and nervous tissue. The results of this work are employed as a forum for the ensuing perfusion experiments.

3. Autoradiographic experiments are made to locate sites of uptake of tritium labelled 5-HT in the brain of Helix pomatia following perfusions with solutions of this substance. These experiments were undertaken to test the hypothesis that a re-uptake mechanism may be partly responsible for inactivation of 5-HT after its presumed release from nerve endings. Such studies were made with special reference to areas of the CNS thought to contain pre-synaptic endings of the GSC (e.g. the buccal ganglia). The possibility that the fine-structural characteristics of labelled axons may represent those of the pre-synaptic endings of the GSC is discussed.

4. Autoradiographic experiments similar to those described in chapter 3 were made in an attempt to find out whether 5-HTP and tryptophan, the precursors of 5-HT, are taken up selectively by specific neurons, 5-HT-containing (e.g. the GSC) or otherwise, or via glial cells.

5. Some electrophysiological data on the function(s) of the GSC of Helix pomatia are presented.

Where relevant, the results of each section are discussed in relation to non-molluscan systems.

Chapter 1

ANATOMY OF THE GIANT SEROTONIN-CONTAINING CELL (GSC) IN THE CEREBRAL GANGLION OF Helix pomatia

Introduction

There is one GSC in each metacerebral ganglion of Helix pomatia. The cell was first noted by Kunze (1921), and some of its electrophysiological and input properties were later described by Kandel and Tauc (1966a, b). Recent interest in the neuron was developed independently by Cottrell and Osborne (1970), when it was shown that both this cell and its homologue in the metacerebral ganglion of Limax maximus contained 5-HT. Because of the ease with which the cell could be identified (the neuron is conveniently located at the proximal end of the internal lip nerve, and furthermore, is the largest cell on the ventral surface of the metacerebral ganglion) it was suggested that it might provide a very suitable system for studying the role of neuronal 5-HT (Cottrell, 1970a). Several recent reports have confirmed this suggestion.

Although most of the data has been obtained for the GSCs in Helix pomatia, homologous cells have been found in the cerebral ganglia of all pulmonate gastropod species so far examined (i.e. Limax maximus, Limax flavius, Arion ater, Arriolimax reticulatus, Archatina fulica, Osborne and Cottrell, 1971; Helix aspersa, Sedden, Kerkut and Walker, 1968; Arion hortensis and Milax budapestensis, Osborne and Cottrell, 1972b). It is likely that the properties of the neuron are common in all these species (Osborne and Cottrell, 1972b).

Several aspects of 5-HT relevant to its proposed transmitter role in the GSCs have been summarised in the introduction. A brief resumé is given here for clarity: 5-HT within the GSCs has been detected by fluorescence histochemistry (Osborne, 1970; Cottrell and Osborne, 1970; Sedden, Kerkut and Walker, 1968; Osborne and Cottrell, 1971), and in the case of Helix pomatia and Limax maximus by bioassay (Cottrell and Osborne, 1970), and by micro-

chromatography of individually dissected neurons (Osborne and Cottrell, 1972a). At least a part of the 5-HT within the GSCs of Limax maximus is bound within granular vesicles of mean diameter 100 nm (Cottrell and Osborne, 1970). The GSCs of Helix pomatia can synthesise 5-HT from 5-HTP both in vitro (Cottrell and Powell, 1971), and in vivo (Osborne, 1972a). Electrophysiological data have shown that each GSC in Helix pomatia forms direct excitatory links with several giant cells in each buccal ganglion (Cottrell, 1970a, b, 1971a, b).

The GSC in Helix pomatia is approximatley 170 μ in greatest diameter. The main axon pathways have been identified by electrophysiological analysis (Kandel and Tauc, 1966a), and by Procion Yellow injection experiments (see Cottrell, 1970a, for a preliminary report).

The present study was undertaken to obtain detailed information on the structure of the perikarya and the axons of the GSCs of Helix pomatia. Serial sectioning was employed to follow the axon branches of the GSCs with the electron microscope, and attempts were made to identify synaptic contacts onto the GSC.

Material and Methods

Specimens of Helix pomatia were obtained from Gerrard and Haig Ltd., East Preston, Sussex.

For light and electron microscopy, nervous tissue was fixed either by perfusion of the intact CNS, or by immersing the dissected pieces in fixative. The fixatives used were as follows: (i) 2.5% glutaraldehyde in 0.2 M cacodylate buffer pH 7.2, (ii) 1% OsO_4 in 0.2 M cacodylate buffer pH 7.2, and (iii) 1% OsO_4 in 0.2 M veronal acetate buffer pH 7.4. Fixation was completed at 4°C. Best preservation was obtained by perfusion with fixative (i) for 2 hr, followed by immersion in fixative (ii) for 1½ hr. After fixation, tissue was dehydrated through a series of acetone-water solutions and embedded in Araldite. Thin sections were stained with lead citrate and uranyl acetate.

Dye injection experiments using Procion Yellow were made using the method of Stretton and Kravitz (1968).

For the histochemical demonstration of 5-HT standard fluorescence microscopical techniques (see Falck and Owman, 1965) were employed. Cerebral ganglia were freeze-dried, exposed to formaldehyde gas, and embedded in paraffin wax. 10 μ sections were examined with a Leitz microscope fitted with a dark field condenser, a BG12 excitation filter, a 530 mμ barrier filter and HBO 200 mercury vapour lamp. The specificity of the observed fluorescence was tested by immersing sections in 0.1 per cent sodium borohydride in isopropanol, a procedure known to deplete specific fluorescence (Corrodi, Hillarp and Jonsson, 1964). In addition, tissues from reserpinized animals were examined. In these experiments snails were injected with 2.5 mg soluble reserpine phosphate for a period of 30 hr. Reserpine has been shown to deplete amines from molluscan nervous tissue by Mirolli and Welsh (1964).

Information on the ultrastructural localization of 5-HT within the GSC of Limax maximus has been obtained by Cottrell and Osborne (1970), who used the technique of Wood (1965, 1966). Osborne (personal communication) has applied the same technique to the GSC of Helix pomatia, and obtained very similar results. Consequently this technique was not employed in the present work.

Data on the nature of possible presynaptic endings of the GSC was obtained from autoradiographic experiments with tritium labelled 5-HT. The experimental procedures are described in chapter 3.

Seven GSC perikarya of H. pomatia have been examined with the electron microscope. In two cases the axons of the GSC were followed with semi-serial sections for a distance of 1 mm through the cerebral ganglion.

Results

(1) General Anatomy

Each GSC is unipolar and has a large nucleus which occupies approximately one quarter of the volume of the perikaryon (Figs. 2-4). The cytoplasm of the neuron fluoresced yellow after tissue had been processed for fluorescence histochemistry (Fig. 4b). The fluorescence was judged specific for 5-HT

using the usual criteria of colour, reducibility with sodium borohydride, and rapid fading on exposure to u.v. light. Prior injection of animals with reserpine reduced the intensity of the fluorescence.

The anatomical pathways taken by the main axon branches of the GSC are shown in Fig. 2. The primary axon from each cell divides into three branches. Two of these pass via the cerebro-buccal connectives to the buccal ganglia, the other runs in the ipsilateral external lip nerve to a complicated system of muscles in the mouth of the animal. The evidence for these axonal pathways has been obtained from electrophysiological analysis (Kandel and Tauc, 1966a; Cottrell, 1970a; see also chapter 5) and from injection experiments with Procion Yellow. There is, furthermore some electrophysiological evidence which suggests that GSC axon branches are present in nerve branches of the cerebro-buccal connectives, and also in nerves leaving the buccal ganglia (chapter 5). The evidence is, however, not complete. These proposed axon branches are therefore not included in the present description.

Within the neuropile of the cerebral ganglion many fine branches arise from the main axon of the GSC which passes to the ipsilateral nerves (Fig. 3). These branches were visualized after injection of the GSC perikaryon with Procion Yellow. They are presumed to be dendrites which receive input onto the GSC.

(2) Fine structure of the GSC perikaryon

The entire surface of the GSC perikaryon is covered with glial cells. Processes from the glial cells invaginate the GSC for distances of 15-20 μ. These glial processes take the form of thin sheets, which are expanded at their distal ends (Figs. 8, 9, 30, 35). Glial cells also cover and extend into the axon hillock and main axon branches of the GSC (see below). One of the most conspicuous types of organelle in the cytoplasm is the small electron-dense vesicle (Fig. 5). These granular vesicles have a mean diameter of 100 nm, and are present throughout the cytoplasm of the GSC, especially

in areas containing granular endoplasmic reticulum. Osborne (personal communication) has found that these vesicles contain electron-dense reaction products after tissue has been processed by the method of Wood (1966). These granules are similar in size and electron opacity to those which are thought to contain 5-HT in the homologous giant serotonin cells of the metacerebral ganglion of the slug, Limax maximus (Gottrell and Osborne, 1970, see Figs. 6, 18), and the granules of the Retzius cells of the leech, Hirudo medicinalis (Rude, Coggeshall and Van Orden, 1969). It is likely therefore that 5-HT is sequestered within the dense-cored vesicles of the GSC.

The cytoplasm of the GSC contains many mitochondria which have simple cristae (Figs. 8, 30). Golgi complexes are scattered in all areas of the cytoplasm (Figs. 5, 30). Other conspicuous structures are ribosomes, situated in the perinuclear region and lysosome-like bodies. The latter organelles are surrounded by membranes, and vary from 0.5 to 5 μ in diameter. Their centres are sometimes composed of a homogeneous material of moderate electron density, which resemble lipid droplets (Fig. 30), but others contain laminated membranes (Fig. 5), or dense-cored vesicles morphologically identical to those present in the cytoplasm. The lysosome-like bodies are concentrated in the area of the GSC cytoplasm which gives rise to the axon hillock (Figs. 3, 4a, 5). In one specimen studied the GSC contained several groups of virus-like inclusions (Fig. 10), which appeared similar to those noted by Frazier et al. (1967) in some central neurons of Aplysia.

The nucleus of each GSC, like those of some other molluscan neurons (see e.g. Rosenbluth, 1963a) occupies a large proportion of the perikaryon. The wall of the nucleus is folded.

(3) Fine structure of the axon hillock and main axon branches

The axon hillock of the GSC tapers gradually over a distance of some 100 μ into the main axon which is approximately 20 μ in diameter (Fig. 3). The shape of the main axon of the GSC varies in cross-section as it runs

deeper into the ganglion (Figs. 11, 12, 19). A prominent feature of the main axon is glial invaginations (Figs. 11, 12, 19). These glial infoldings are more extensive than those on the cell body. Serial sections show that the invaginations are membrane-bound sheets or fins which run for distances of 10-50 μ along the axon. These glial infoldings are present on each major axon branch.

In a previous study on the abdominal ganglion of Aplysia, Coggeshall (1967) noted glial foldings on the axon hillocks of large ganglion cells which were so extensive that the axon was divided into smaller processes, which re-united at a distance from the cell some. This phenomenon, which is an extreme example of a trophospongium (Holmgren, 1901), does not appear to occur to the same extent in the GSC or other large nerve cells of Helix pomatia so far examined with the electron microscope. The initial portion of the axon of the GSC contains many deep glial infoldings, but these have not been observed to cross or divide it.

The axoplasm of the GSC is markedly different from the cytoplasm of the cell body. The point at which this change in the character of the cytoplasm takes place is the beginning of the axon hillock. In this region the occurrence of large numbers of lysosome and pigment granules terminates abruptly, as if some barrier separated them from the axon (Figs. 3, 4a). Beyond this point the main axon branches of the GSC contain less, and fewer types of, organelle than the perikaryon.

The most conspicuous structures in the axoplasm are neurotubules (diameter 25 nm) which run parallel in the long axis of the axon (Figs. 11, 12, 16). The number of neurotubules which may be seen in any cross-section of the main axon of the GSC approaches 10,000. The neurotubules originate at the beginning of the axon hillock, and have been found running within all the GSC axon branches that it has been possible to follow with the electron microscope. It is not known however if any individual neurotubule runs continuously to a particular axon branch of the GSC, or whether neuro-

tubules originate at different points within the axons.

Besides neurotubules, only two other types of organelle were recognizable within the axons of the GSC. These were small mitochondria and dense-cored vesicles identical in appearance to those present in the GSC perikaryon (Figs. 13, 14). Occasionally cell processes containing electron-dense structures were seen to protrude several microns into the main axon of the GSC (Fig. 14, inset). The significance of these structures is not clear. It is possible that they are processes of glial cells.

The main axon of each GSC gives rise to many fine processes which interweave the neuropile of the cerebral ganglion in which the perikaryon is located (Figs. 13, 14). It was difficult to study these because any loss of serial sections meant loss in known continuity with the GSC. (On the otherhand, the loss of one or two sections did not hinder following the larger, and straighter running main axon). The axoplasm of the fine branches was similar to the main GSC axon. Neurotubules and filaments were seen along their length (Fig. 13). On several occasions, fine branches were seen to end in club-like expansions containing a few dense-cored vesicles similar to those present in the GSC perikaryon. However no concentrations of dense-cored vesicles, nor membrane thickenings were observed that indicated that such branches were pre-synaptic endings of the GSC. The main axon branches of the GSC were successfully followed for a distance of approximately 1 mm through the cerebral ganglion. At this level, the main axons of the GSC supplying the buccal ganglia were up to 8 μ in broadth. Special note is made here of the consistent nature of the axoplasm from the hillock to the fine processes in the cerebral neuropile, especially with regard to the presence of neurotubules and dense-cored vesicles.

(4) Synapses onto the GSC

In gastropod molluscs, synapses similar to those present in vertebrates are rarely seen (e.g. Gerschenfeld, 1963; Coggeshall, 1967). It is not known if this is because points of synaptic transmission are rare in gastropod neuropile, or if it is because many synapses are not characterized by membrane

thickenings on either side of the cleft. It is likely that some of the axon processes in gastropod neuropile which contain large concentrations of vesicles are presynaptic terminals which do not have obvious membrane specializations (Coggeshall, 1967).

During extensive studies on the cerebral ganglion of H. pomatia no points of contact onto the GSC were observed which closely resembled the type of synapses commonly seen in the vertebrate central nervous system (see Gray, 1971). On the other hand, many structures were seen which satisfied several of the criteria for a synapse; namely two opposed neuronal processes, one presynaptic and filled with mitochondria and vesicles or granules, the other (the GSC axon) postsynaptic and containing few inclusions at the point of apposition.

No synapses are present on the perikaryon or the initial region of the axon trunk of the GSC. Structures, presumed to represent synapses on the basis of the above criteria, were first seen some 200 μ from the perikaryon. In this region, small groups of fine axons come into contact and run parallel with the axon of the GSC (Fig. 12). Some of these axons contain large numbers of agranular vesicles of mean diameter 50 nm; others contain larger (diameter 100-200 nm) electron-dense granules (Fig. 12, inset). The latter were morphologically similar to those contained within the cell processes occasionally seen protruding into the axon of the GSC (cf. Fig. 14, inset). Each GSC sends fine branches into the adjacent groups of axons. These branches appear to be synapsed onto by the axons containing the vesicle types described above (Figs. 16, 17). Glial processes are found amongst the axons, and in some sections (e.g. Figs. 16, 17) were seen to fold over and isolate anatomically the groups of axons running against the GSC process. This was the case where more than one group was present against the same GSC axon (Fig. 12), and between groups on different GSC axons studied.

Other areas of possible synaptic contact onto the GSC were seen at a greater distance from the perikaryon, in the region where the cells gives rise to many fine branches within the neuropile of the cerebral ganglion (see Fig. 3)

Fig. 20 shows an axonal process which makes contact with the GSC in this branching region. The process contains a mixed population of clear centred and dense-cored vesicles, some of which abut the membrane apposed to the GSC axon. Other possible incoming pre-synaptic endings in this region contained large numbers of either clear centred vesicles (mean diameter 50 nm), or dense-cored vesicles (diameter 50-150 nm) (Fig. 14).

(5) The structure of presumed presynaptic endings of the GSC

The available data indicate that some presynaptic endings of the GSC are situated in the neuropile of the buccal ganglia, and others are situated on, or close to, muscles in the lips of the animal (Cottrell, 1971a; see also chapter 5). Both of these areas are approximately 1 cm away from each GSC perikaryon. It is not practicable to follow axons by serial sections with the electron microscope over such a distance. Consequently it was not possible to study any structures which could be definitely identified as presynaptic endings from the GSCs.

However, in the following chapter it will be shown that a small proportion of axons and nerve endings in each buccal ganglion neuropile, and in the region of the lip musculature of Helix pomatia, selectively accumulate tritium labelled 5-HT both *in vivo* and *in vitro*. In consequence to this it is postulated that re-uptake is a mechanism of inactivating 5-HT after liberation from nerve endings *in vivo*. Some of the evidence for re-uptake of 5-HT as a mechanism of its inactivation is based on the finding that exogenously supplied serotonin is taken up by nerve endings in the close proximity of neurons in the buccal ganglia which are thought to receive synaptic inputs from the G. C (see chapter 3). Furthermore these endings contain dense-cored vesicles which are morphologically identical to those found in the perikarya of the GSCs. Figs. 42-46 show axon profiles in the neuropile of the buccal ganglia of Helix pomatia which have become radioactive after exposure of the ganglia to 5-^{3}HT. It is of interest that the dense-cored vesicles in these labelled endings are similar to those in the GSC not only with respect to the dimensions of the

outer membrane and core, but also with respect to their electron opacity.

If some of the labelled axons in the buccal ganglion neuropile are presynaptic endings from the GSC, an important question is whether they are associated with typical synaptic structures. No synaptic clefts with pre- and postsynaptic thickenings were observed in association with the labelled endings. However, synaptic structures with membrane thickenings were rarely seen in the neuropile of the buccal ganglia. The problem is the same as that of interpreting synaptic areas on the branches of the GSC in the cerebral ganglion which has been described above.

Discussion

Many anatomical features of the GSC of Helix pomatia are very similar to those of giant neurons described in other gastropod species. Such features have been extensively discussed in earlier works. This is the case regarding the large size and irregular shape of the nucleus (Rosenbluth, 1963a; Coggeshall, 1967), the penetration of the neuron's surface by glial cells (Amoroso, Baxter, Chiquoine and Misbet, 1964; Coggeshall, 1967), the numerous branches arising from the main axons of the GSC in the central ganglion (Bullock and Horridge, 1965; Benjamin and Ings, 1972), and the nature of many of the organelles and inclusions of the GSC cytoplasm (Coggeshall, 1967; Schmekel and Wechsler, 1968).

The following discussion, however, deals with certain more specific structural aspects of the GSC which can be related to its transmitter content and function.

1. Ultrastructural localization of 5-HT

It is thought that the small dense-cored vesicle (mean diameter 100 nm) sequesters 5-HT in the GSC perikaryon for several reasons: (1) These vesicles are the only structures of the GSC which are not noticeable in neighbouring cell bodies that lack 5-HT. (2) The cores of these vesicles react positively to the technique of Wood (1965, 1966), which has shown to be specific for amines in molluscan nervous tissue (Cottrell and Osborne, 1969a). (3)

Treatment of ganglia of the related species Limax maximus with reserpine, a drug which depletes amines from molluscan nervous tissue (Mirelli and Welsh, 1964), and lowers the level of 5-HT in the GSCs (Cottrell and Osborne, 1970), abolishes the positive reaction of these vesicles using the Wood technique.

Vesicles morphologically identical to those thought to sequester 5-HT in the GSC perikaryon were seen in all axon branches of the GSC that were followed with the electron microscope. These vesicles are also present in large numbers in certain axonal processes which are selectively labelled by tritiated 5-HT, and which are found in the neuropile of the buccal ganglia and in the lip musculature (see chapter 3). Both these areas are thought to contain presynaptic endings of the GSC (Cottrell, 1972a; see also chapter 5).

It is thought that Golgi complexes are responsible for forming at least some of the dense-cored vesicles found in gastropod neurons (e.g. Coggeshall, 1967, 1971; Bullock and Horridge, 1965). It is likely that this is also the case in the GSC perikaryon, for occasional Golgi cisternae in this cell appeared to be 'budding off' dense-cored vesicles (see Fig. 5, inset). No Golgi complexes were observed in any axon of the GSC examined with the electron microscope.

If the central ganglia of Helix pomatia are exposed in vivo to tritium labelled 5-HTP, 5-HT-containing cells are selectively labelled (see chapter 4). In the case of the GSCs, the radioactivity in the axons of the GSCs may in part be radioactive 5-HT transported from the perikarya. In relation to this, Osborne and Cottrell (1970) have shown that ligatured peripheral axons of Helix pomatia show accumulation of specific yellow fluorescent material on the proximal side of the ligature, suggesting a proximo-distal transport of 5-HT along nerves.

Thus the available evidence suggests that some of the dense-cored vesicles seen in the main axons and presumed endings of the GSC are packages of 5-HT which originated in the cell bodies. If this is the situation, there is

evidently a close parallel to the binding and transport of noradrenaline which is thought to occur in noradrenergic neurons of the sympathetic nervous system (see Dahlström, 1971).

However, Cottrell and Osborne (1970) have obtained evidence using the technique of Hood (1965) that under some conditions, perhaps associated with season, a proportion of the lysosome-like bodies in the GSC of Limax maximus contain 5-HT. In relation to this point, it is interesting to note that a proportion of the lysosome-like bodies in both the GSC of Limax maximus (Osborne, 1970) and Helix pomatia contain dense-cored vesicles which are identical to those thought to bind 5-HT in the cytoplasm of the GSCs. The significance of the lysosome-like bodies, 5-HT-containing or otherwise, within the GSCs is not clear. These organelles vary greatly in size and content. It is not known whether such variations represent different stages in the development or decline of one functional population or whether there are several functionally different types within each GSC. The problem of the function of the lysosome--like inclusions found in molluscan neurons has been discussed by Nolte, Breuckler and Kuhlmann (1965), and Zs.-Nagy and Borovyagin (1972).

It is perhaps also interesting to compare the vesicles present in the GSC, with those present in an identifiable dopamine-containing cell. This large neuron is present in the left pedal ganglion of Planorbis corneus. Its fluorescence characteristics (method of Falck, 1962) indicate that it contains dopamine (Marsden and Kerkut, 1970), and electrophysiological data have shown that some of the dopamine may serve both excitatory and inhibitory transmitter roles (Berry and Cottrell, 1973). Preliminary examination has shown that this cell contains numerous dense-cored vesicles (Fig. 7). These vesicles vary in external diameter from 100 nm to 500 nm, which is a different situation from the relatively constant diameter of dense-cored vesicles present in the GSC. Work is in progress to determine whether such vesicles sequester dopamine.

2. Synapses onto the GSC

No structures resembling synapses were seen on the perikaryon of the GSC.

This finding is consistent with studies on other large neuron perikarya in gastropods (e.g. Rosenbluth, 1963; Coggeshall, 1967); although axo-somatic synapses have been described on small neurons present in the procerebrum of Helix pomatia (Zs.-Nagy and Sakharov, 1969, 1970).

Furthermore, no structures were identified on the branches of the GSC within the cerebral ganglion, nor were any structures observed in association with the possible presynaptic endings of the GSC in the buccal ganglia and lip musculature, which could definitely be interpreted as synapses by the established morphological criteria derived from studies in the vertebrates. It is unlikely that such absence was due to the fixation and staining procedures employed, because such synapses were occasionally seen within the neuropile of both the buccal and cerebral ganglia of each preparation examined (see Fig. 15).

However 'typical' synaptic structures are only rarely seen in many gastropod species (Gerschenfeld, 1963; Rosenbluth, 1963; Coggeshall, 1967; Gorman and Mirolli, 1970). In the present work therefore, some structures which did not possess all the features of a typical vertebrate synapse were interpreted as being possibly synaptic in nature.

Many axonal processes containing agranular vesicles (mean diameter 50 nm), or granular vesicles (50-150 nm diameter), or both, make close contact with fine branches arising from the main GSC axons within the neuropile of the cerebral ganglion. The agranular vesicles are morphologically similar to those thought to sequester acetylcholine in the mammalian brain (De Robertis et al., 1962; Whittaker, 1971). Pharmacological studies have shown that the GSC falls into the category of neurons named D cells by Tauc and Gerschenfeld (1961) because they are depolarized by acetylcholine. All excitatory synaptic inputs onto the GSC are blocked by tubocurarine (Kandel and Tauc, 1966a). It is therefore possible that the axonal processes which contain large numbers of agranular vesicles and which abut each GSC are points of excitatory input onto the GSCs. Each GSC also receives inhibitory synaptic input which is mimicked by glutamic acid (Cottrell, Macon and Szczepaniak, 1972; Szczepaniak and Cottrell, 1973), and it is possible that some of the axons which come into contact with the GSCs

and which contain granular vesicles may be sites of inhibitory input and release glutamic acid.

3. GSC function in relation to its geometry

Because the axons of the GSCs, which pass to the buccal ganglia form monosynaptic links with several neurons in these ganglia (Cottrell, 1970), and because each GSC receives synaptic inputs within the cerebral ganglion (Kandel and Tauc, 1966a; Cottrell, Macon and Szczepaniak, 1972), it is likely that the GSCs have, at least in part, an interneuronal function. However electrophysiological analysis has shown that one other main axon of each GSC runs peripherally in the external lip nerve (Kandel and Tauc, 1966a) to muscles in the region of the mouth (see chapter 5). Selective stimulation of either GSC causes a marked increase in the gross electrical activity recorded extracellularly from the muscles, and this effect is mimicked by 5-HT applied to the muscles (Figs. 58, 59). If this effect is produced *in vivo* by 5-HT released from the GSCs directly onto the muscles, then not all the branches of the GSC have an interneuronal role. It is perhaps interesting that certain neurons in Aplysia and Tritonia have been suggested as having both interneuronal and motor functions (Hughes and Tauc, 1961; Willows and Hoyle, 1969; Coggeshall, 1971).

The anatomical and geometrical features of the GSC are compatible with the electrophysiological findings and proposed functions of the cell. This is especially the case with the branches of the GSC in the cerebral ganglion, which very probably receive the external synaptic inputs. On the other hand, it was not possible to identify with certainty the endings of the GSC, but because certain axonal processes in the buccal ganglia and lip musculature which contain granular vesicles identical to those in the GSC soma selectively take up labelled 5-HT (chapter 3), it is likely that some of these processes are presynaptic endings of the GSC. Precise data on the anatomy of the endings of the GSC will however only become available by selective marking of all the axon branches of the GSC and their subsequent examination with

the electron microscope. This is not possible with current mapping techniques. An attempt is being made to achieve this by injecting labelled compounds (e.g. leucine, 5-HTP) into the perikarya of the GSCs, and then examining the nervous tissue by electron microscopic autoradiography.

4. A comparison of the GSC with other 5-HT-containing neurons

Perhaps the only comparable situation in which a study has been made of an identifiable serotonergic neuron is that of Rude, Coggeshall and Van Orden (1969), who examined the Retzius cells of the leech. In these neurons, 5-HT is bound within granules which resemble the GSC dense-cored vesicles in that they are of the same size (mean diameter 100 nm) and contain cores of similar electron opacity. However they differ in that their cores are often eccentrically placed against the membrane of the vesicle, whereas the GSC vesicle cores tend to be round and more centrally placed.

In the mammalian nervous system there is some evidence that 5-HT may be localized within dense-cored vesicles (Whittaker, 1971; Jaim-Etcheverry and Zieher, 1969; Bloom and Costa, 1971; Pelligrino de Iraldi, Guedet and Suburo, 1971). However it is also evident that a great deal of heterogeneity must be considered in the granulated vesicles of different nerve endings, even in the granulated vesicles of the same nerve (Pelligrino de Iraldi, Guedet and Suburo, 1971).

Thus, there is not a single morphological type of vesicle that can be used to identify the presence of 5-HT in neurons of different phyla. Furthermore different vesicle forms may bind 5-HT within different neurons, or different parts of the same neuron, within the same species.

Fig. 2. Diagram (approximately to scale) showing the arrangement of the axons of the right GSC of Helix pomatia. The pathways taken by the axons of the left GSC are the mirror image of this picture. The GSC perikarya is located on the ventral edge of the cerebral ganglion (c). Some of the endings of the GSC are present in the buccal ganglion (B), others are present in muscles of the lip of the animal (LM). Near the peripheral end of the external lip nerve (ELN) is situated a small ganglion (LG) which contains several hundred small neurons. Axon branches from the GSC leave this ganglion via several small nerves. CBC, cerebro-buccal connective, MLN, middle lip nerve, ILN, internal lip nerve, CPC, cerebro-pedal connective.

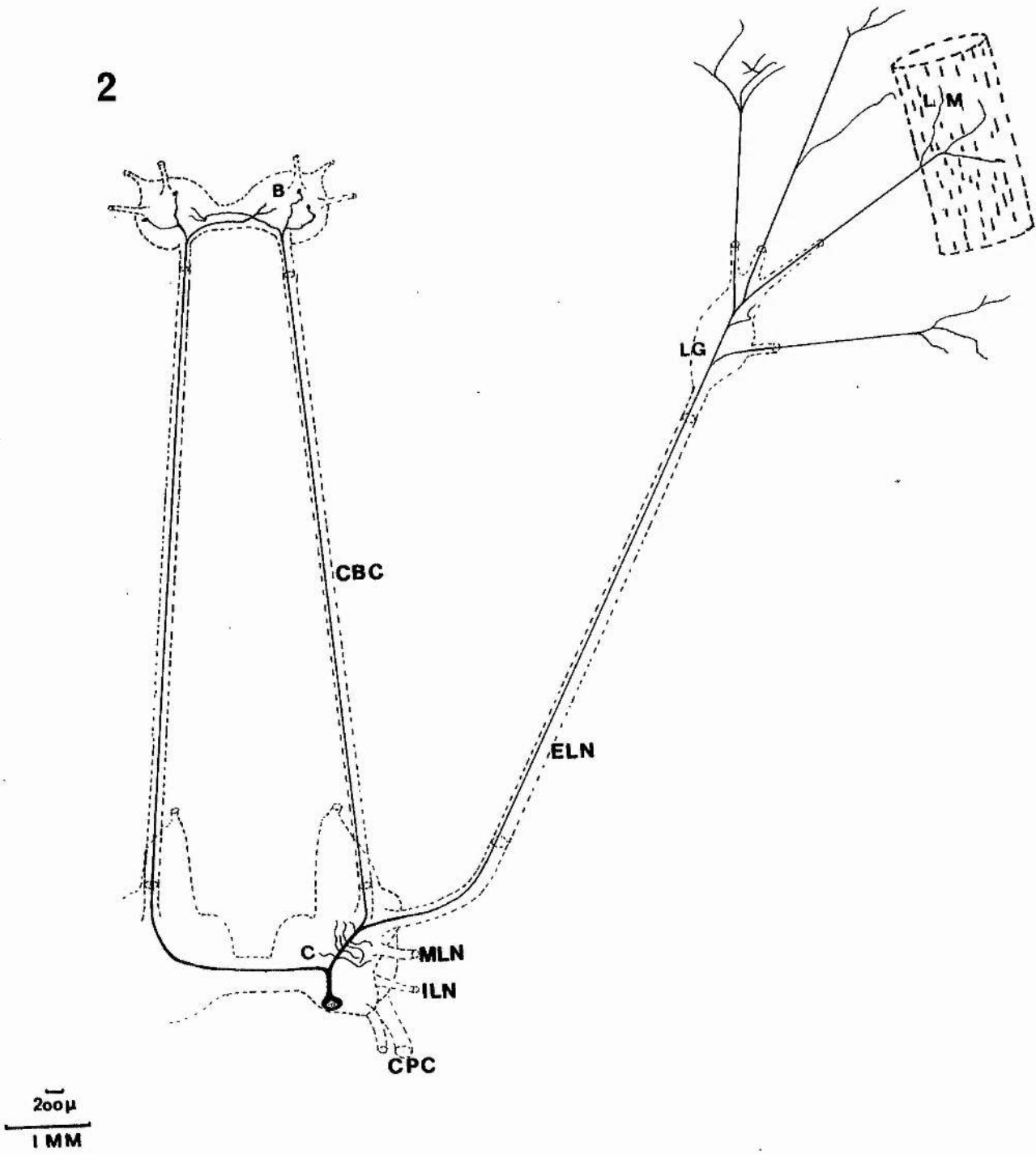

2
B
CBC
LG
L M
ELN
C
MLN
ILN
CPC
200μ
1 MM

Fig. 3. A diagram showing the GSC and its axon branches within the cerebral ganglion. The size and arrangement of the perikaryon and main axon branches are relatively constant in each GSC and are reconstructed approximately to scale, but the fine branches arising from the main axon vary in their arrangement and are shown as they might appear in a typical preparation. The dotted lines at the edges of the GSC perikaryon show the surface of the ganglion beneath the connective tissue capsule. A large proportion of the GSC surface is close to the blood sinuses which cover the surface of the ganglion (see also Fig. 25). The numbered lines and areas represent the levels of subsequent electron micrographs. N, nucleus of the GSC. H, axon hillock. G, glial cells. L, Lysosome-like bodies. CC, cerebral commissure. CBC, cerebro-buccal connective. IELN, ipsilateral external lip nerve.

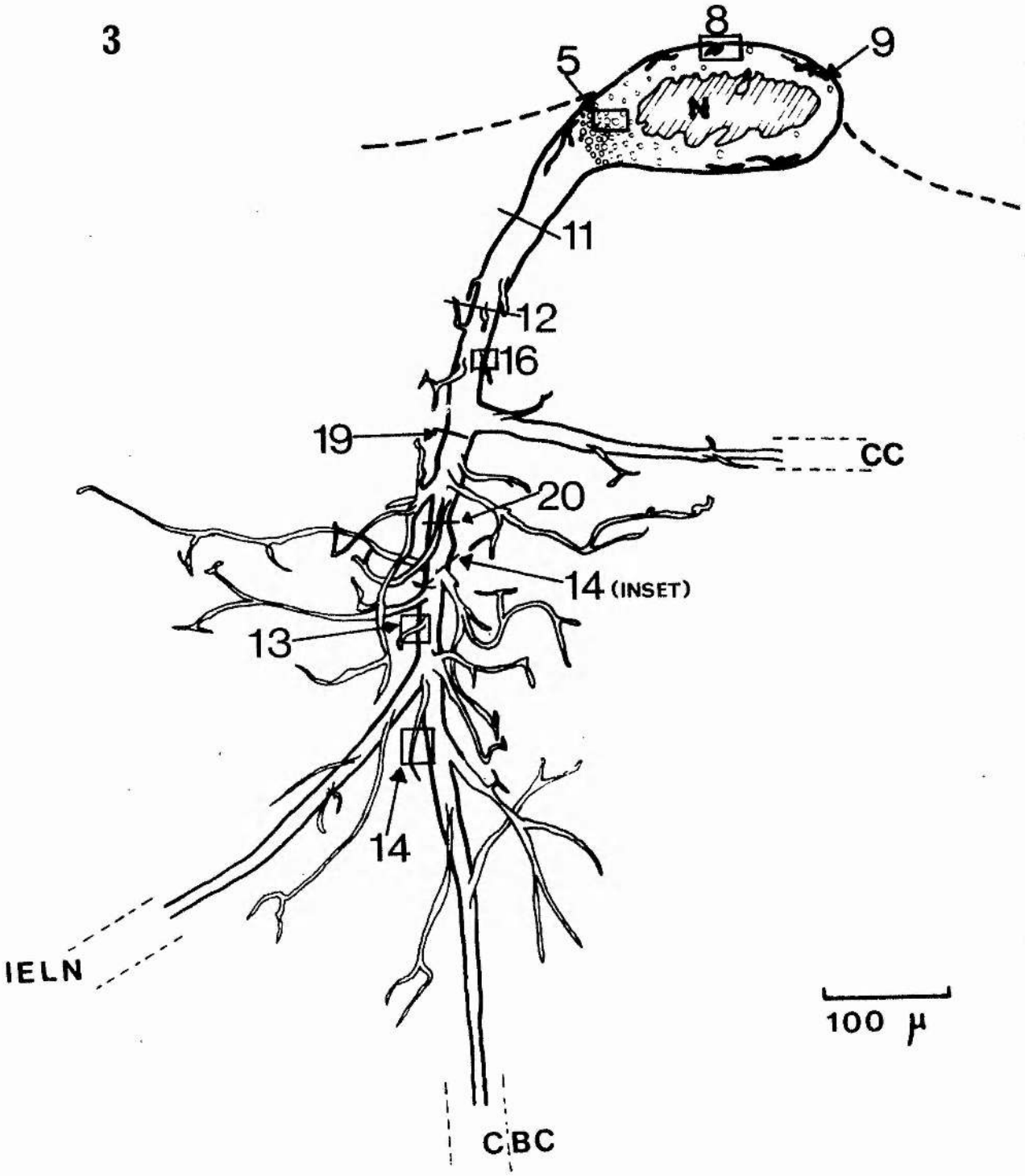
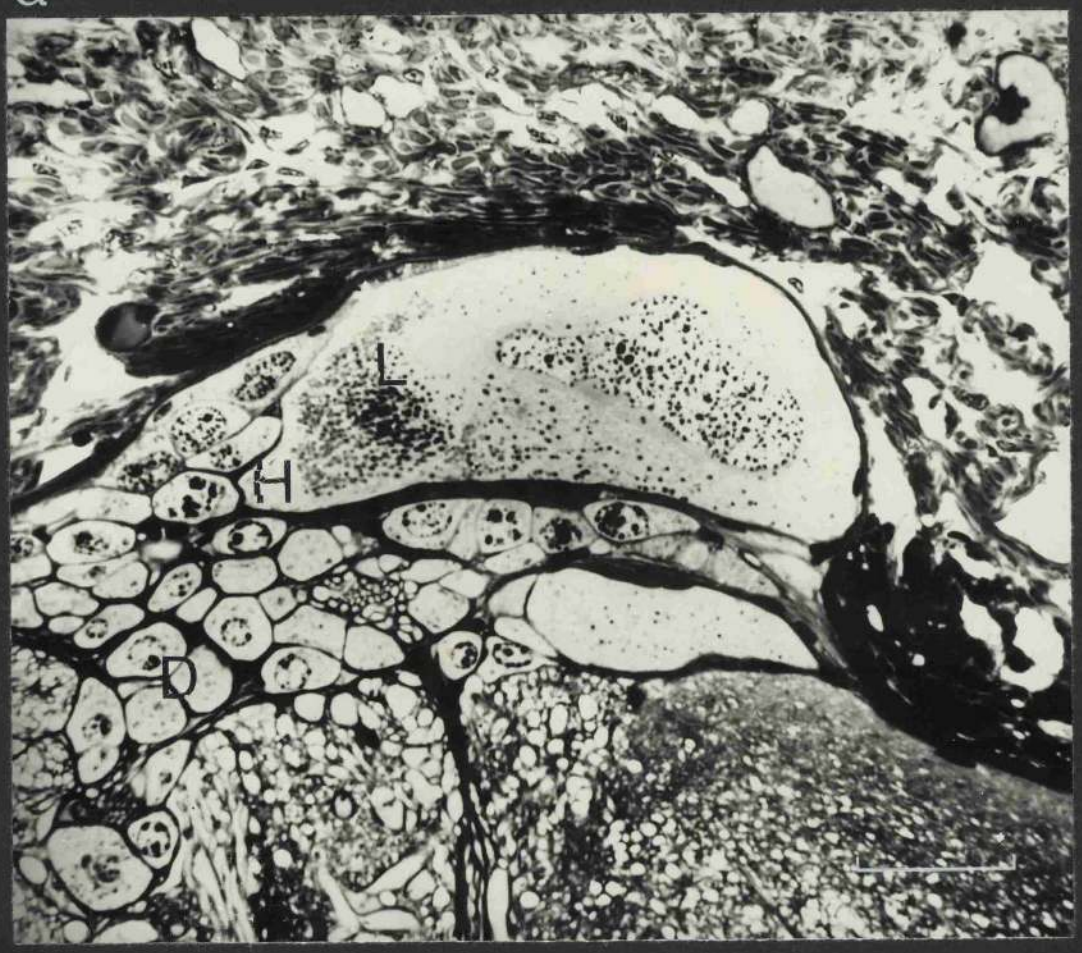

3
8
9
5
N
11
12
16
19
CC
20
14 (INSET)
13
14
IELN
100 μ
CBC

Fig. 4a. Light micrograph of a section through a GSC of Helix pomatia. The material has been fixed in glutaraldehyde and osmium, and embedded in Araldite. The section (2 μ thick) has been stained with Toluidine Blue. Lysosome-like bodies (L) are concentrated in the deeper regions of the GSC cytoplasm, but do not extend into the axon hillock (H). The small neurons (D) are in the same position as those which fluoresce specifically for catecholamines (cf. Fig. 4b). Scale is 70 μ.

Fig. 4b. A section through the metacerebral portion of a cerebral ganglion which has been processed by the histochemical method for the demonstration of biogenic amines. The micrograph shows a section through the GSC, the cytoplasm of which is fluorescing specifically for 5-HT. The nucleus (N) of the GSC does not fluoresce. A cluster of smaller-sized neurons (D), and a region of cerebral ganglion neuropile (P) fluoresce specifically for the catecholamines dopamine and/or noradrenaline (cf. Fig. 4a). The photograph was reproduced from Agfachrome 50L (Agfa-Gevaert) transparency film. Scale is 70 μ.

a



b

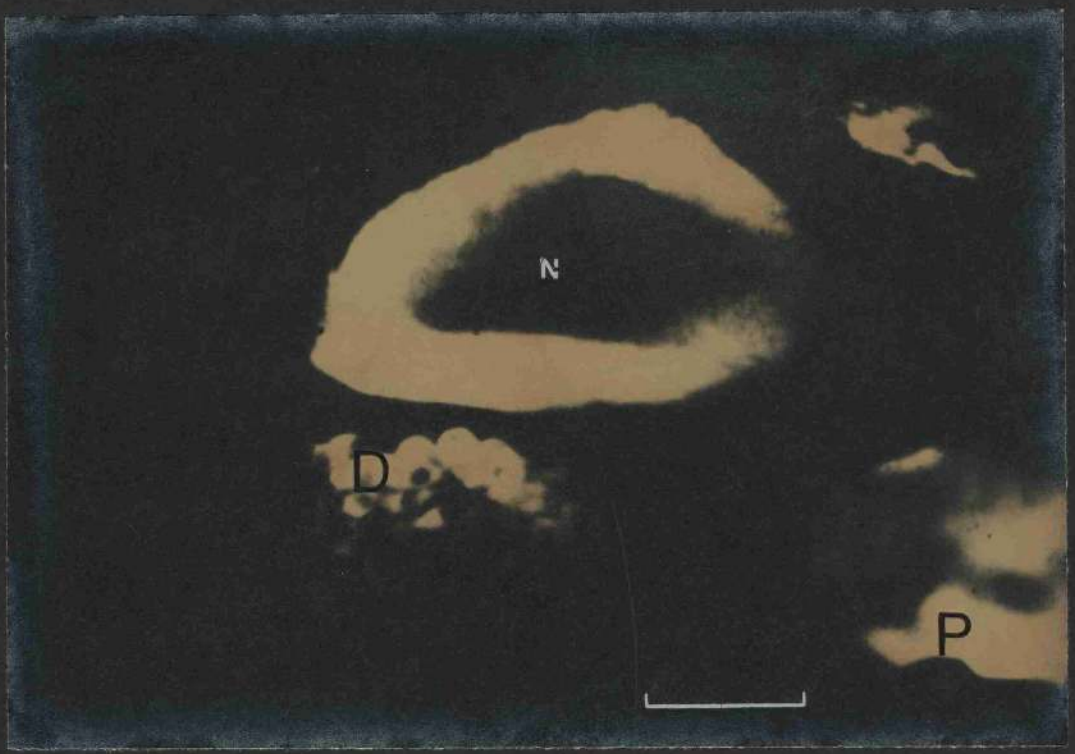
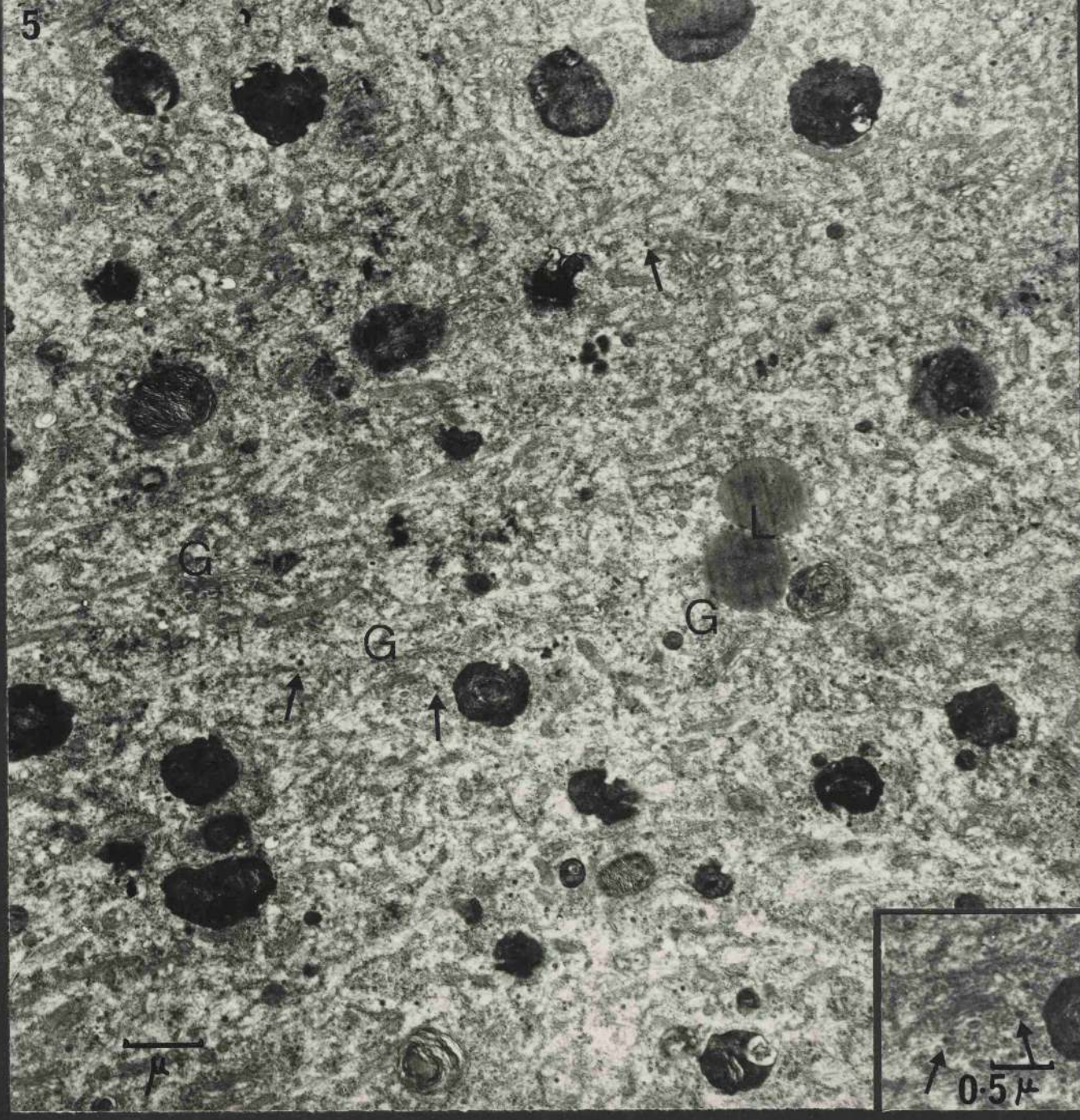
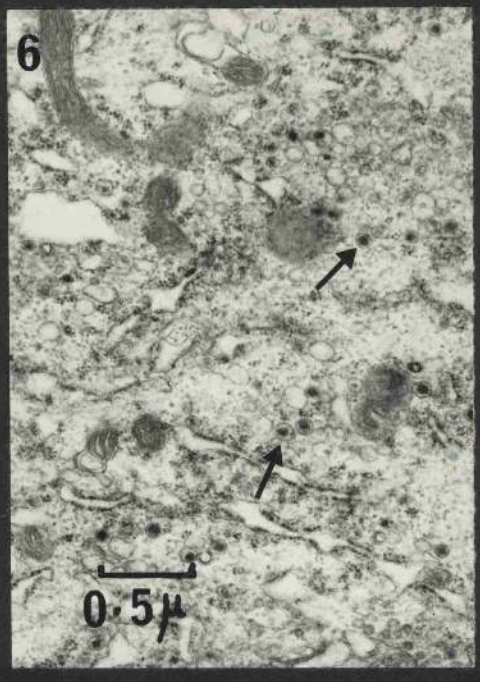


Fig. 5. Electron micrograph showing some inclusions of the GSC cytoplasm The section shows a part of the axon hillock of the cell. Some of the lysosomes of this region contain lamellate membranes, others resemble lipid droplets (L). Arrows point to some of the dense-cored vesicles. Several Golgi structures (G) are present; one of these is illustrated at higher magnification in the inset because of its apparent close association with dense-cored vesicles. M, mitochondria.

Fig. 6. Electron micrograph showing some inclusions of the cytoplasm of the GSC of Limax maximus (Micrograph supplied by N.N. Osborne). Arrows point to some of the dense-cored vesicles. See also Fig. 18.

Fig. 7. Electron micrograph of a section through the giant dopamine-containing neuron in the left pedal ganglion of Planorbis corneus. This cell contains large numbers of dense-cored vesicles which vary in external diameter. It is possible that such vesicles sequester dopamine.


5
G
G
G
L
μ
0·5μ


6
0·5μ

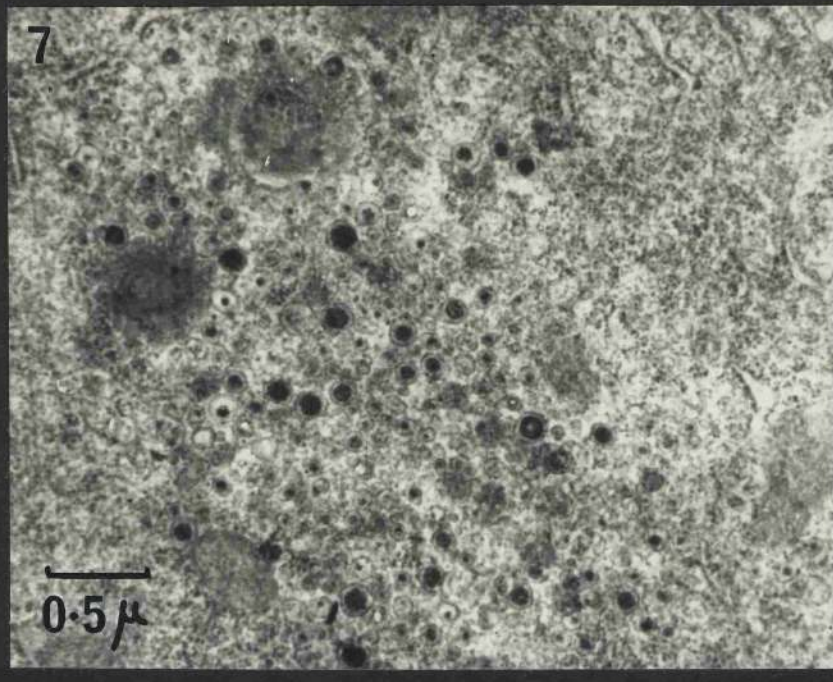
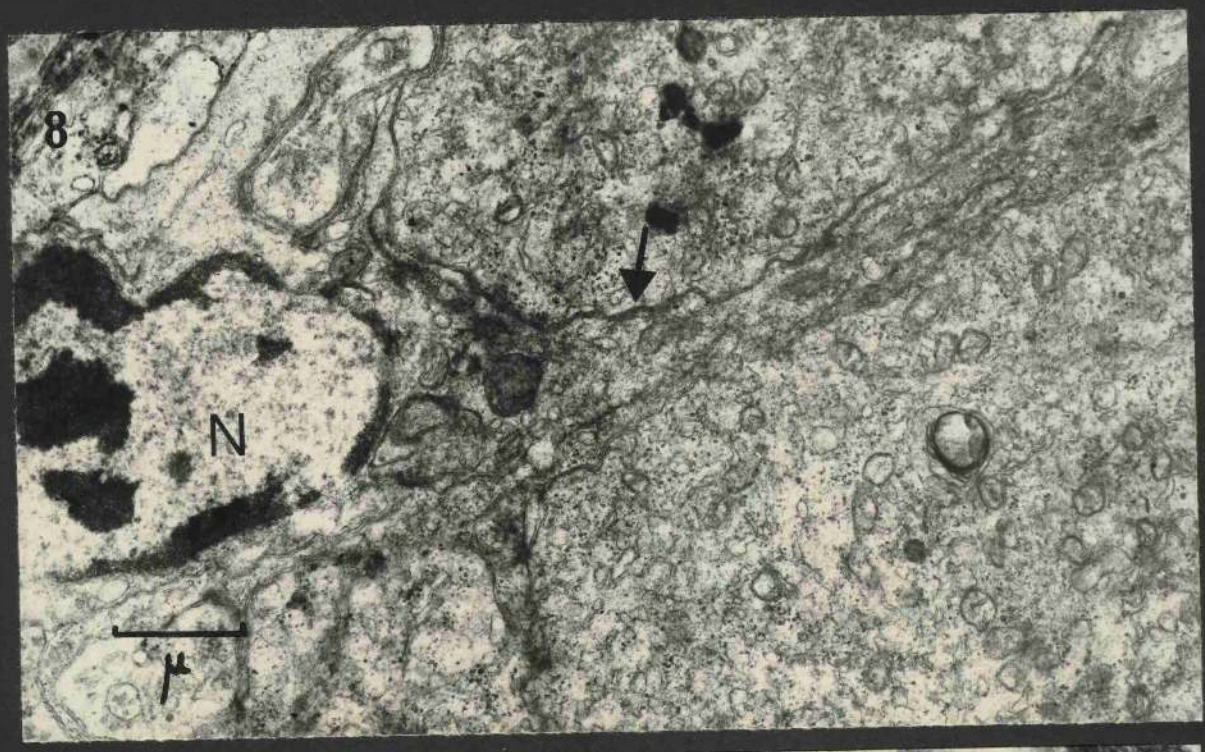

7
0·5μ

Fig. 8. Electron micrograph of a section through the outer edge of a GSC of H. pomatia. A glial cell (nucleus, N) invaginates the GSC cytoplasm. Arrows point to glial cell infoldings.

Fig. 9. Oblique section through the ventral edge of a GSC (see Fig. 3). This micrograph illustrates the sheet-like nature of the glial infoldings. The ends of the glial foldings are expanded into club-like processes (arrows). L, lysosome-like bodies.

Fig. 10. Electron micrograph of a section through the cytoplasm of a GSC showing a group of three virus-like inclusions. Each one is composed of 12 subunits arranged in identical order, i.e. rows of 2, 3, 4, and 3 such subunits. These structures were seen in only one cell out of those examined.


8
N
μ


9
L
μ

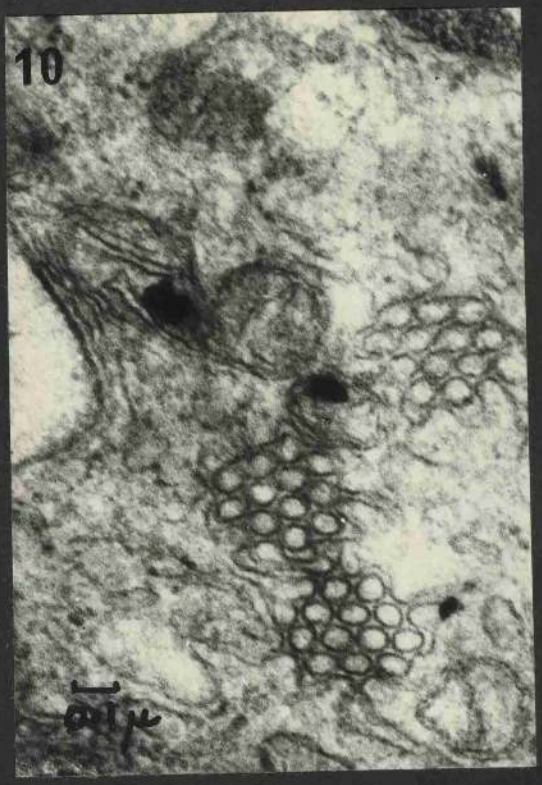

10
0.1μ

Fig. 11. Electron micrograph showing a cross-section of the main axon of a GSC of H. pomatia at the level indicated in Fig. 3. A glial cell (nucleus, N) sends processes into the GSC. The empty areas of cytoplasm (arrows) may be fixation artefacts, or they may serve some unknown function. The axoplasm contains large numbers of microtubules.

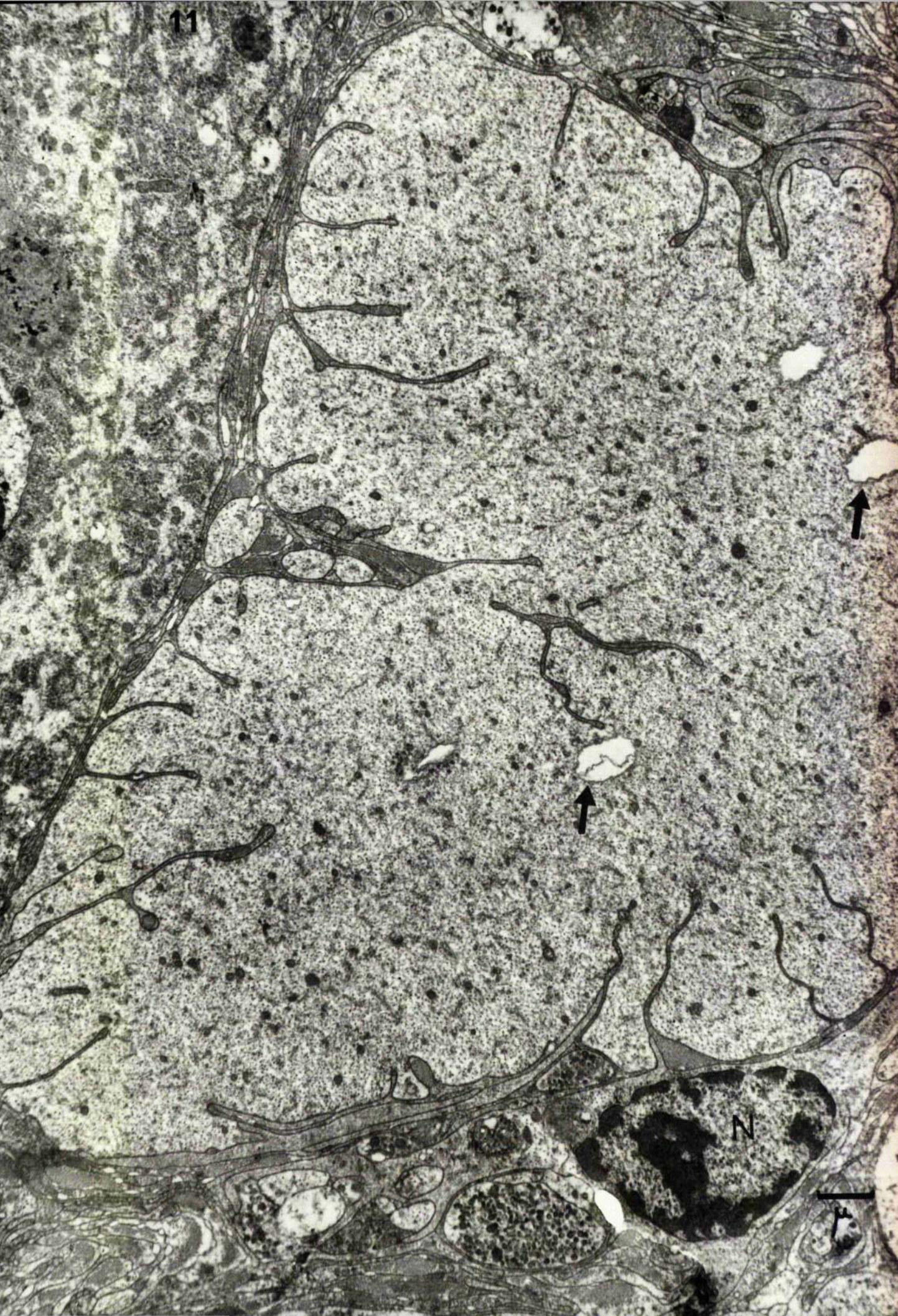

11
N
μ

Fig. 12. Electron micrograph showing a cross-section of the main axon of the GSC at the level indicated in Fig. 3. The micrograph illustrates the nature of the glial infoldings, and shows two regions (A,B) of presumed synaptic connections onto the GSC. The axoplasm contains numerous microtubules (see inset) and small mitochondria. The arrow points to an empty area of cytoplasm, which may be an artefact of fixation, or which may serve some unknown function. The inset shows possible synaptic endings onto the GSC. One of these (S) contains clear synaptic-type vesicles, the other electron-dense structures.

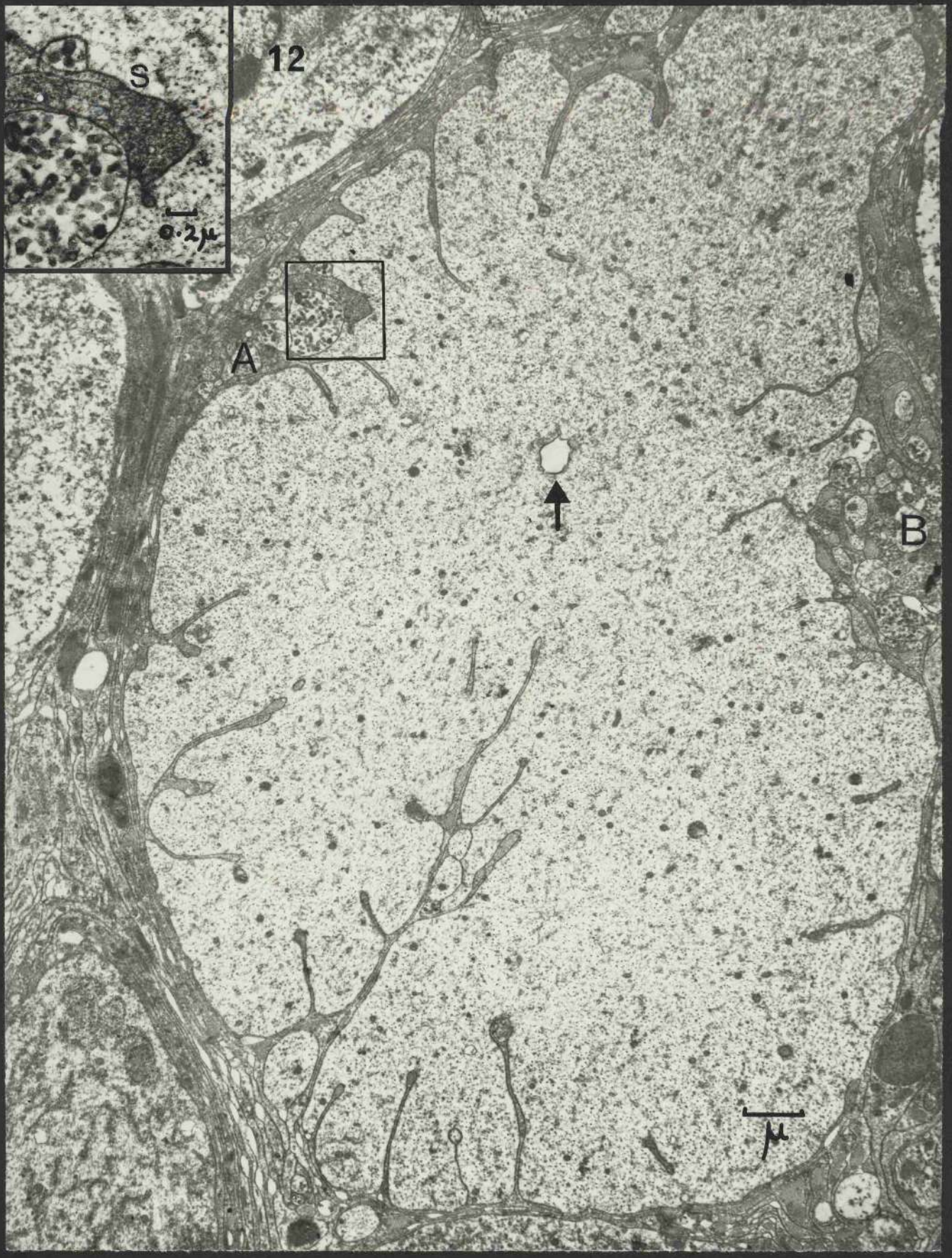

12
S
0.2μ
A
B
μ

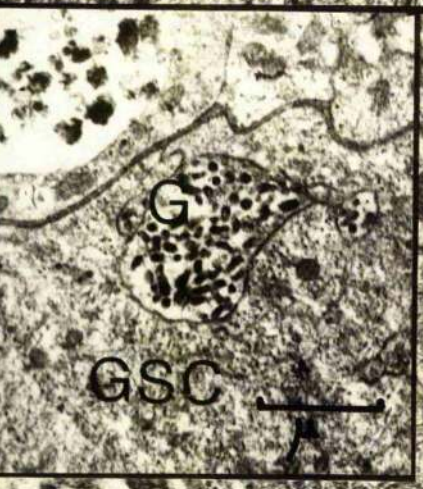
Fig. 13. Micrograph showing a fine branch of a GSC of H. pomatia whose end is expanded, and which contains a few dense-cored vesicles (arrows). The filaments and tubules running lengthwise in this axon branch indicate that it is sectioned longitudinally. There are relatively few organelles in the axoplasm of this branch compared with that of the neighbouring nerve endings within the neuropile, which contain many types of vesicles.

The quality of fixation shown in Figs. 13 and 14 is inferior to that of the GSC cell body, because these areas are deep within the neuropile, at a greater distance from the surface of the ganglion.

Fig. 14. Micrograph showing another example of fine branching axons (asterisks) leaving the main axon of a GSC. These fine branches enter and intermingle with the neuropile of the cerebral ganglion. Axon profiles containing electron-transparent vesicles (S), dense-cored vesicles (D), or a mixture of vesicles (M) come close to these fine branches. The arrow points to a dense-cored vesicle within an axon of the GSC which is morphologically similar to those present in the cell body of each GSC.

The inset shows a structure (G), which appears to be a finger or spine penetrating the GSC and cut in oblique section. The structure contains osmiophilic granules similar to those which abut the GSC in areas of presumed synaptic contact (see Fig. 12).


GSC
13
μ


G
GSC
μ

S
M
D
GSC
14
μ

Fig. 15. Electron micrograph showing a structure in the neuropile of a buccal ganglion of H. pomatia which is interpreted as being a synapse. The presumed presynaptic ending (P) contains numerous vesicles, whereas the presumed postsynaptic axon (F) is relatively empty. The membranes are thickened at the presumed locus of the synapse. This structure closely resembles synapses in the mammalian brain.

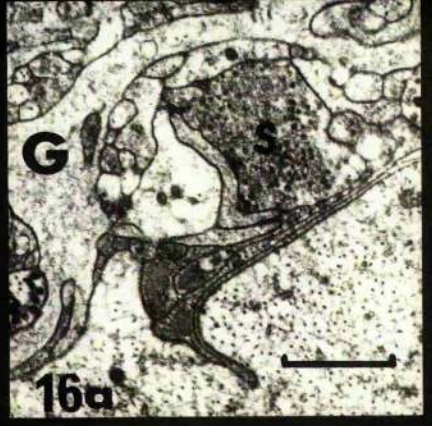
Figs. 16-17. Electron micrographs showing semi-serial sections through a structure which is interpreted as being a possible synapse(S) onto a fine axon branch of the main GSC axon. A possible arrangement of the components is shown diagrammatically in Fig. 17, where the numbers indicate the levels of section shown in Figs. 16a, b, c, d. It is suggested that the fine branch from the GSC axon makes contact with the process abutting the presumed presynaptic ending(s) which contains large numbers of agranular vesicles (Fig. 16c). Glial material (G) appears to encapsulate the structure. The scale in each Figure is one micron.

Fig. 18. A portion of the GSC cytoplasm of a preparation from Limax maximus processed by the method of Wood (1965) for the demonstration of amines. There is close agreement between the distribution and size of the reacting sites, which are the only electron-dense structures visible, and the distribution of vesicle cores shown in Fig. 6 (Micrograph supplied by N.N. Osborne).

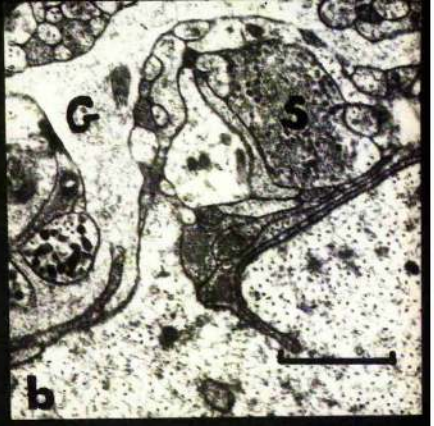
Fig. 19. Electron micrograph showing a glial cell which lies against the main axon process of a GSC (see Fig. 3). The glial cell sends processes into the GSC axon. N, nucleus of glial cell.

Fig. 20. The axonal process (M), which contains a mixed population of vesicles, makes close contact with an axon of the GSC (see Fig. 3). Some of the vesicles lie tightly against the membrane of the GSC. The arrow points to an infolding of the GSC membrane, which is at the locus of the possible synapse. Neurotubules can be seen in cross-section in the GSC axoplasm.

15



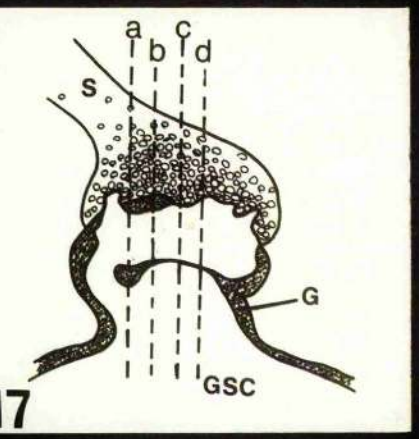
16a



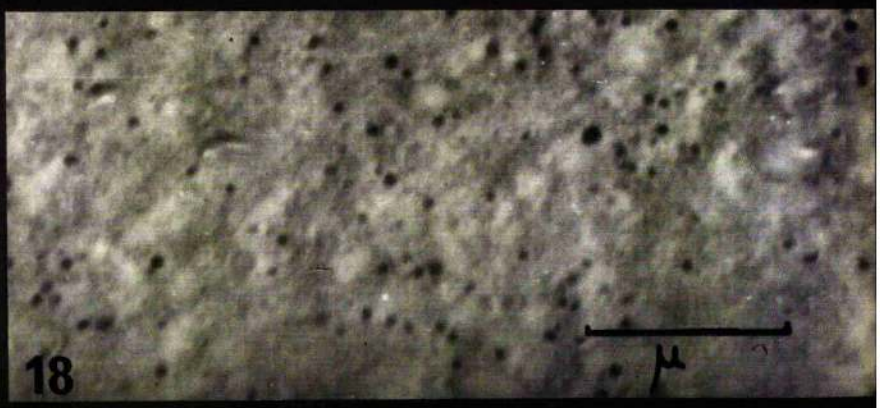
b

c

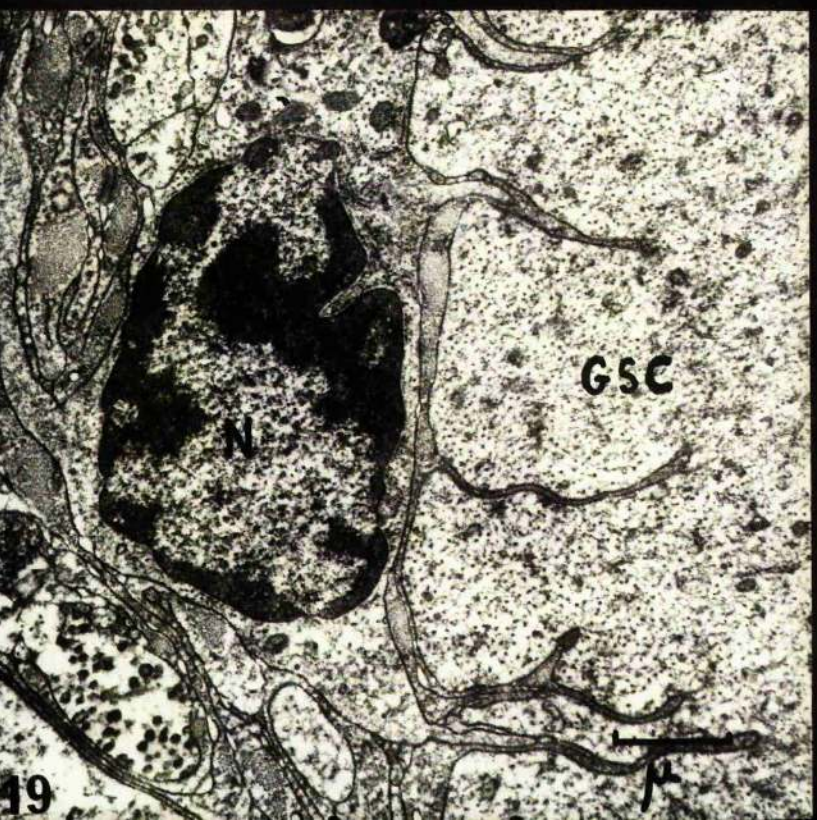
d



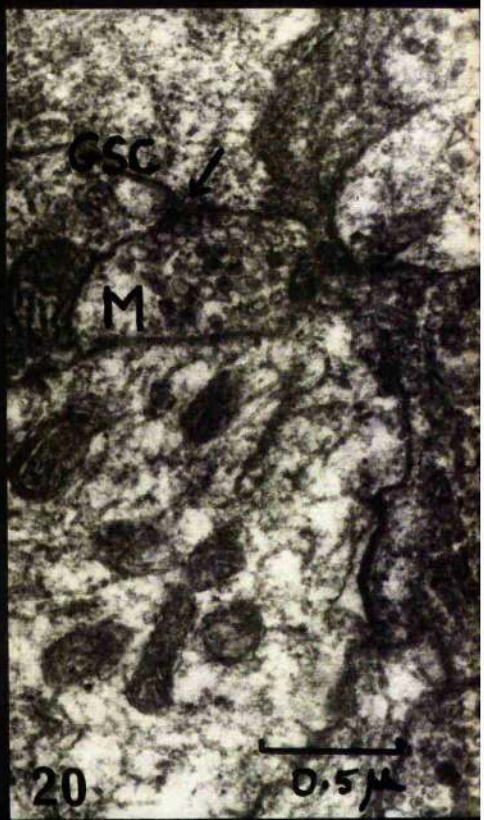
17



18



19



20

Chapter 2

THE BLOOD SUPPLY TO THE CENTRAL NERVOUS SYSTEM OF Helix pomatia

Introduction

Pulmonate molluscs have an open vascular system. The heart receives blood from sinuses, and pumps it through arteries to different parts of the body where it passes back into the sinuses.

The anatomy and the physiology of the heart of many pulmonate species are well known, (e.g. Helix pomatia, Schwartzkopff, 1954), but there is little information available concerning the arterial and sinus systems. In this chapter, an attempt is made to describe in detail the blood supply to the central nervous system of Helix pomatia. This study was necessary for the ensuing perfusion experiments used to study the uptake of radioactive substances from the blood system in vivo.

Materials and Methods

(1) Microscopic anatomy

After minimal dissection, arteries were injected with rubber latex (Revultex, Revertex Ltd.) or perfused with injection ink (Pelikan). The latex (natural latex in 5% ammonia) was diluted with distilled water to 30% of the original concentration, and coloured by the addition of a small amount of Vulcafor Fast Blue dye (ICI Ltd.). The ink was diluted to 30% in snail ringer (Wong, 1960). The progress of each tracer into the various branches of the arterial system was followed visually under a binocular microscope. By injecting in turn each small artery while others were ligatured, it was possible to trace the final paths taken by the blood to the sinuses. The preparations injected with latex were fixed for 6-12 hr in alcoholic Bouin's solution (pH 3.0). Because latex is transformed to rubber at low pH, fixation caused the latex to solidify, thus leaving a tough

replica of the arterial system which withstood thorough dissection. In some cases latex injected specimens were dehydrated (alcohol series) and cleared (xylene), to give whole preparations which showed well the relation of the arterial supply to the various central ganglia.

Ink injected specimens were not so useful for tracing the general blood system, since the slightest rupture during dissection caused leakage of ink which masked details. Instead these preparations were fixed for 5-15 hr in 0.2 M phthalate buffer pH 4.2 containing 6% glutaraldehyde and 2% acrolein, dehydrated (methanol series), cleared in toluene and embedded in paraffin wax. Serial sections (10 μ) were cut through the region being studied and stained with toluidine blue. Because the ink retained in the blood system provided good contrast under the light microscope, it was possible from these sections to trace the finest arteries.

(2) Electron microscopy

Whole cerebral ganglia or pieces of the sub-oesophageal ganglionic complex were processed for electron microscopy as described in the previous chapter i.e. tissues were fixed for 1½-2 hr in 1% OsO_4 in 0.2 M cacodylate buffer pH 7.2. Some specimens were first fixed for 2-3 hr in a mixture of 10 ml 2% glutaraldehyde plus 2 ml 0.3 M cacodylate buffer pH 7.4, washed for 15 min in 0.12 M cacodylate buffer, and then postfixed in 1% OsO_4 in 0.2 M cacodylate buffer pH 7.2 for 1 hr. All fixation processes were completed at 4°C. The pieces of tissue were then dehydrated through a series of acetone-water solutions and embedded in Araldite. Sections were cut on a LKB Ultrotome, stained with lead citrate and uranyl acetate and examined with an AEI EM6B electron microscope.

In some experiments, tracers were introduced into the vascular system prior to fixation to study the precise relationships of the blood-nervous tissue interface. 20% Ferritin (Koch-Light) in Meng's snail saline, or 30% injection ink (Pelikan) in saline were injected at low pressure via a small bore plastic cannula into the artery supplying the area of nervous tissue

being investigated. Preparations were left in this state for 2 min, 20 min, 2 hr, 24 hr, after which the connective tissue sheath covering the injected area of nervous tissues was gently manipulated with forceps. Any resulting contraction of the sheath was taken as evidence that the preparation was still alive; the tissue was then fixed.

(3) Pharmacological tests

The anterior aorta was dissected out and suspended in a 10 ml organ bath. A thread tied to one end of the aorta was attached to a balanced aluminium lever and isotonic recordings were made using a kymograph.

Other experiments were made in which the aorta was suspended from a cannula, and the rate of perfusion of saline through the aorta measured with the photo-transistor drop chamber coupled to a rate recorder. Results were recorded on a kymograph. The cerebral and sub-oesophageal ganglia were perfused in the same manner, but here the appropriate arterial branches were tied off to avoid escape of saline into the capillary beds not under investigation.

In each case the following drugs were added to the perfusing or bathing saline to give final concentrations from 10^{-8}-10^{-3} g/ml: dopamine hydrochloride, l-nor-adrenaline bitartrate, l-adrenaline hydrochloride, 5-HT creatinine sulphate, acetylcholine bromide.

Results

1. Anatomy of the blood supply to the central nervous system

The central ganglia are supplied by branches of the anterior aorta. With the exception of the supply of the buccal ganglia, these branches arise at the termination of the aorta within the sub-oesophageal ganglionic complex.

Figs. 21 and 22 show the general arrangement of the arterial system supplying the central nervous system. The main anterior aorta follows closely the intestinal nerve and enters the nerve ring at the ventral edges of the right parietal and visceral ganglia. The artery then runs anteriorly between the right parietal and pedal ganglia, bends ventrally between the anterior

edges of the two pedal ganglia, and runs in a posterior direction between the pedal nerves. This vessel finally supplies the middle of the foot (Fig. 21-24, fa).

The principal branches of the aorta arise where it curves between the pedal ganglia (Fig. 24). First a single artery, the ventral buccal artery, runs forward in the mid-line, bends dorsally and proceeds to the buccal mass. Besides supplying the ventral buccal musculature, this artery opens into cavities within the buccal mass. According to Schmidt (1916), the blood here has a mechanical function; it braces the radular membrane. The next vessels arise anterior to the ventral buccal artery, and are paired. These arteries (the anterior arteries) branch laterally over the pleural ganglia, and run dorsally outside the cerebro-pedal connectives. At the level of the cerebral ganglia, each anterior artery gives off small branches which supply the cerebral ganglia (Figs. 21, 23, cr, ci), and the large and small tentacles. The third major arteries, the right and left anterior foot arteries, arise from the aorta where it curves ventrally between the pedal ganglia. Each vessel follows closely the first so called cutaneous pedal nerves (Schmalz, 1914), and eventually supplies the anterior and lateral foot musculature.

The supply to the sub-oesophageal ganglia is shown in detail in Fig. 24. There is some individual variation in the arrangement of the smallest capillaries; the diagram illustrates the general features. Each ganglion of the complex is supplied by capillaries from the main arteries described above. The visceral and parietal ganglia receive several small branches from the aorta. These ramify into the connective tissue sheath, eventually forming an almost continuous blood-filled space which lies very close to the surface of the nervous tissue. Small vessels continue from this space within the connective sheath covering the pallial and intestinal nerves. The nervous tissue itself, within the connective tissue, is completely avascular. In a similar fashion the pleural and pedal ganglia are supplied by branches from the right and left

anterior arteries, and also from the aorta. Again the capillaries form a blood filled space over the cell rind of each ganglion. The various nerves which leave the pedal and pleural ganglia normally have an accompanying small artery. Blood permeates through the spaces within the connective sheath into the sinus that surrounds the ganglia.

Fig. 23 illustrates the arrangement of the vessels supplying the cerebral ganglia. Near the point at which the cerebro-buccal connective and external lip nerves leave each ganglion, one or sometimes two branches are given off from the anterior arteries. If there is one branch it bifurcates to give vessels which supply the dorsal and ventral surfaces of the ganglia. If there are two branches the first to arise goes directly to the dorsal surface, while the other supplies the ventral surface. Both the dorsal and ventral vessels run over the junctions of the procerebrum and metacerebrum, giving off capillaries which ramify over the whole cerebral ganglia and extend along the nerves leaving it. The nature of the final supply to the nervous tissue is identical to that in the sub-oesophageal ganglia. This is illustrated diagrammatically in Fig. 25. Blood fills the spaces within the connective tissue sheath, and so forms an almost continuous blood space over the nervous tissue. Finally blood passes out through the connective sheath into the body--cavity sinuses.

Several vessels arise from the aorta between the heart and the lateral branch of the aorta (see Schmidt, 1916) but these are not shown because they do not supply nervous tissue. The lateral branch (Fig. 21) principally serves the crop and salivary glands, but it also eventually supplies the buccal ganglia. This is shown in detail in Fig. 26.

2. Electron microscopy

With the electron microscope it is possible to observe the vascular channels which pass through the connective tissue sheath of each ganglion. The vascular channels are bounded by a thin luminal endothelium which is often folded (Fig. 27). Besides endothelial cells the connective tissue sheath contains

muscle cells (Figs. 29, 31), small groups of axons (Figs. 31, 32), collagen (Figs. 27-32), and several other cellular types (see Sanchis and Zambrano, 1969; Rogers, 1969).

At the surface of the nervous tissue, where the vessels join together to form a blood space (Fig. 30), there are three layers between the blood and the neurons of each ganglion. First there is a lining endothelium. This is similar to that lining the vessels in the connective tissue, but here the cells and their nuclei are long and thin (Fig. 30). Second there is a connective layered composed of collagen fibres with interspersed muscle cells (Figs. 28, 35, 36), and third there are glial cells (Figs. 30, 33-35).

Of the three layers the endothelium is the most continuous, varying between 0.1 μ and 1.0 μ in thickness. The connective tissue layer, on the other hand, differs a great deal in form and appearance. In some places it is composed of tightly packed collagen fibres which run in different directions in different layers (Fig. 28). In other places the fibrils appear loosely interwoven in a lightly staining background matrix. This matrix sometimes contains large electron opaque granules (Fig. 33), which are very similar to those present in the glio-interstitial elements in Glossodoris (Nicaise, Pavans de Ceccatty and Baleydier, 1968). In still other parts of the ganglion, such as that covering the ventral edges of each CSC, collagen appears to be almost lacking (Fig. 34).

The glial cells normally occupy a monocellular layer between the fibrous connective tissue and the central neurons. The membranes of the glial cells are much folded, and frequently send finger-like processes into the nerve-cell cytoplasm (Fig. 30). Another feature of the glial cells is the apparently empty vacuoles which are present between their membrane foldings (Figs. 33, 35). These may be caused by the leaching out of some substance during the histological processing. They do not however appear to be an artifact of bad fixation, because the surrounding tissue seems quite normal. Sattelle and Lane (1972) have described similar 'empty' areas of tissue between glial cells surrounding

the central nervous tissue of Limnea stagnalis.

The introduction of ink into the vascular system shows clearly the anatomical boundaries of the blood spaces. Figs. 33, 34 illustrate the arrangement of the blood with respect to the connective tissue, glial and neurons in different parts of the cerebral ganglia. These micrographs also show that particles of colloidal carbon which have a mean diameter of 50 nm cannot pass outside the blood spaces. There was no indication of carbon particle passage through the capillary endothelium 24 hr after the injection of ink into the blood system.

The experiments with ferritin (molecular diameter 10 nm), on the other hand, indicated that a particle with this diameter or less could move freely into the extracellular spaces (Figs. 35, 36). 20 min after the introduction of this substance into the blood system, particles had crossed both the capillary endothelium and the fibrous connective tissue coat (Fig. 36). Thereafter some of the tracer entered for a short distance the extracellular space between the glial cells but not into the glial cells themselves. At 24 hr after injection particles were not observed within glial, nerve or muscle cells.

3. Pharmacological tests

Catecholamines, 5-HT and acetylcholine caused changes in the length of the anterior aorta when they were added to the bath in which it was suspended. The results are summarised in Table 1. It appeared that these effects were taking place along the whole length of the aorta, since isolated sections of it responded in the same manner.

Table 1

Effect of drugs on the isolated anterior aorta

Substance	Threshold bath concentration necessary to cause an effect (gm/ml)	Effect
5-HT	10^{-5}-10^{-6}	relaxes
Acetylcholine	10^{-6}	relaxes
Dopamine	10^{-5}	contracts
Noradrenaline	10^{-5}	contracts
Adrenaline	10^{-4}	contracts

In order to test whether these drugs affected the diameter of the aorta, as well as its length, the aorta was perfused. Typical results are shown in Fig. 37. In these experiments the concentration of drugs necessary to produce threshold effect was ten to one hundred times lower than in the bath experiments. Unlike the relaxing effect of acetylcholine on the aorta suspended in the bath, the perfused vessel was caused to constrict.

The results of perfusing the isolated brain and the cerebral ganglia (Fig. 37) were similar to those obtained by perfusing the anterior aorta. However, the rate of flow of the perfusing saline through the connective tissue sheath was slower than through the aorta.

The physiological significance of the dilating and constricting effect of amines on the aorta and connective tissue capillaries are not clear at present. No evidence was obtained for the presence of amines in either aorta wall or in the connective tissue of each ganglion by fluorescence histochemistry (method of Corrodi and Jonsson, 1967).

Discussion

The most comprehensive works on the pulmonate circulatory system are those of Schmidt (1916) and Rold (1924) on Helix pomatia, and Boer and Lever (1959) on Ferrissia shimekii. However, of these reports only the last pays special attention to the vessels supplying the central ganglia.

A comparison between the arrangement of the vessels in Helix and those in the Basommatophoran pulmonate Ferrissia reveals several differences. First the anterior aorta enters the nerve ring of Helix ventrally between the visceral and right parietal ganglia, whereas in Ferrissia it enters ventrally beneath the left parietal ganglia. Second, a sinus praeganglionaris, which in Ferrissia is a swelling of the aorta in the anterior region of the sub-oesophageal complex, from which the ventral buccal artery arises, does not exist in Helix. Third, the supply to the left and right paired ganglia is relatively asymetrical in Ferrissia, but with one or two minor exceptions (i.e. there may be one or two cerebral arteries on either side) it is symmetrical

in Helix. The difference between Helix and Ferrissia may be true for all the Stylommatophoran and Basommatophoran species.

The present work is apparently the first to describe a discrete blood supply to the buccal ganglia of a pulmonate. Although Schmidt (1916) showed two small arteries accompanying the salivary ducts, each of which branched over the dorsal buccal mass, he did not indicate that they also branched ventrally around the buccal mass and supply the buccal ganglia. Furthermore, Boer and Lever (1959) working on Ferrissia stated that "blood vessels supplying the buccal ganglia were not found."

One point is made here regarding the terminology of some of the anterior vessels. Previous authors (e.g. Schmidt, 1916; Schmalz, 1914) have named the principal branches of the aorta by the first organ supplied by that branch. This is sometimes misleading, for the first organ is often only supplied indirectly via a particular branch. Such is the case with the cerebral ganglia. The two branches from the anterior aorta which run outside the cerebro-pedal connectives have in previous works (e.g. Schmidt, 1916) been referred to as the arteriae cerebralis. These vessels however principally supply the large and small tentacles and the areas of the body wall surround them. The right vessel also supplies the penis. The cerebral ganglia are supplied by two very small, but discrete, branches from these large arteries in this work.

The original main branches of the aorta have been called the right and left central anterior arteries, for it is the anterior region of the animal which they principally serve.

The experiments involving the injection of ink and latex as tracers for study at the light microscope level show clearly the final blood paths around the nervous tissue. It is evident that these paths are present as an almost continuous blood sinus which is separated from nervous tissue by three layers. These are (i) a luminal endothelium, (ii) a fibrous connective tissue layer which is mainly collagen and (iii) glial cells. In a previous interpretation of Aplysia, Bales (1921) stated that the "whole of the nervous system, both

central and peripheral, is surrounded by a sheath of connective tissue between which and the nervous tissue lies a blood sinus." On the other hand Chalazonitis (1961), after injecting the central ganglia of Aplysia with Janus Green and examining these ganglia under a dissecting microscope, described capillaries that passed over the surface of neurons within the substance of the nervous tissue. Because these workers relied on light microscopy, which does not have sufficient resolution for distinguishing thin tissue layers between blood and nervous tissue, Coggeshall (1967) examined ink-injected ganglia of Aplysia with the electron microscope. He found that the ink filled many blood vessels and large sinuses throughout the sheath which were lined with endothelial cells. Furthermore he found many carbon particles free within interstices of the sheath that were not lined by endothelial cells. The latter situation appears different from that found in the present work for Helix, where all ink-filled spaces were lined with endothelium. The reason for this difference is not clear at present. Coggeshall (1967) did not however examine closely the relationship between ink-filled sinuses and the nervous tissue.

The almost continuous blood space around the nervous tissue in each central ganglion reflects some of the unique characters of the gastropod central nervous system. Because of the extreme changes in shape that take place between the animal's contracted and extended positions, the epineural sheath contains many muscle cells (Scholte, 1957) which allow for adjustment of ganglion shape and nerve length in the various positions (Rosenbluth, 1963b). The almost continuous blood space between the sheath and nervous tissue allows each to move relatively independently of each other, and thus avoid rupture or displacement of the nervous tissue e.g. during rapid withdrawal of a snail into its shell, nerve sheaths running from the central ganglia to peripheral tissues actively contract, while the nerve is passively coiled into a spiral within the sheath. Furthermore the close association of arteries with nerves leaving the various ganglia allows easy distribution of the blood in all positions, at the same time supplying the nerves themselves. It is of interest that Nolte (1967) has shown that sites of neuro-

secretory activity are present along some cerebral NERVES neurons of Lymnaea stagnalis. One such nerve is the median lip nerve, which in Helix is closely accompanied by the inferior tentacular artery. Thus it is possible that neurosecretory products from the central ganglia may be channelled directly to specific target areas which are supplied by the arteries.

The ultrastructure of the cellular types of the epineural sheath have been described in Helix aspersa by Rogers (1969) and Fernandes (1966, 1971), in Cryptomphallus aspersa by Sanchis and Zambrano (1969), in Viviparous contectus and Lymnaea stagnalis by Settelle and Lane (1972), and in Aplysia californica by Coggeshall (1967). Other aspects of the sheath such as its histochemistry (Fernandez, 1966) have also been investigated. The general conclusions of these workers is that the connective tissue sheath is either an important barrier between the haemolymph and the central nervous system which is completely avascular (Rogers, 1969; Sanchis and Zambrano, 1969), or is involved in active transport of ions (Fernandez, 1966). The results of the present work would indicate that a slight readjustment of this idea is necessary, for blood is channelled directly from the branches of the anterior aorta to the surface of the nervous tissue, and is then passed out through the sheath to the sinuses. Furthermore, the results of the experiments with ferritin indicate that there is little or no diffusion barrier for material of less than 10 nm diameter between the blood spaces, and the extracellular glial spaces which surround the neurons. A similar situation appears to be true for the crustacean Carcinus maenas (Abbott, 1970, 1971, 1972). Settelle and Lane (1972) have provided other evidence, by using electrophysiological techniques, that the nerve sheaths of Viviparous and Limnea are not involved in active transport of sodium or potassium ions. Their results indicate that these ions have unrestricted access to neuronal surfaces.

The perfusion experiments suggest that acetylcholine, and certain amines which are important in the physiology of gastropods (see Cottrell and Leverack, 1968) may also play a role in the control of the anterior blood system in

H. pomatia. Three possibilities for the control of blood passage to discrete capillary beds (besides changes in the heart rate) are: (1) nervous control of artery diameter (for a description of these nerves see Schmals, 1914), (2) control by active substances in the blood, and (3) changes in the state of muscular contraction of the epineural sheath. With regard to the latter, numerous groups of axons are present in the epineural sheaths of the central ganglia (Figs. 31, 32). Furthermore, Benjamin and Pent (1968) have described myoneural junctions in the connective tissue sheath of the osphradial ganglion of Planorbarious corneus, and Rogers (1969), and Fernandez and Fernandez (1972) have described myoneural junctions in the epineural musculature of Helix aspersa. Acetylcholine and/or biogenic amines could play a part in these possible control mechanisms.

In summary, it appears that there is no barrier in Helix pomatia, or other gastropods, between blood and nervous tissue similar to the blood-brain barrier in the mammalian brain. Passive diffusion through open extracellular channels appears to be the mechanism for the exchange of ions, and other substances between the blood and extra-neuronal fluid. Thus the organisation of the blood supply to the brain of Helix pomatia would appear to provide a very suitable preparation for perfusion experiments.

Fig. 21. Diagram showing the general blood supply to the central ganglia of Helix pomatia in dorsal view. The nervous tissue in this and subsequent diagrams is black; the arteries are outlined. The buccal mass (B) is shown extended and stretched forward for clarity. The buccal ganglia are supplied by vessels (bl, br) which follow the salivary ducts (SD). These vessels arise from a lateral branch of the aorta, al. The anterior aorta (aa) branches within the sub-oesophageal complex. A median vessel (bv) supplies the ventral buccal mass, and two lateral branches (la, ra) supply the tentacles, and the central ganglia via the cerebral arteries (cl, cr). B, buccal mass. SD, salivary duct. SG, salivary gland. ST, small (inferior) tentacle retractor muscle. T, large tentacle retractor muscle. aa, anterior aorta. al, lateral branch of aorta. bl, left buccal artery. br, right buccal artery. bv, ventral buccal artery. cbc, cerebro-buccal connective. cl, left cerebral artery. cpc, cerebro-pedal connective. cplc, cerebro-pleural connective. cr, right cerebral artery. el, external lip nerve. fl, left foot artery. flm, left lateral foot artery(s). fm, middle foot artery. fr, right foot artery. frm, right lateral foot artery(s). il, internal lip nerve. ml, median lip nerve. p, pedal nerves. ra, right anterior artery. rp, right pallial nerve. t, tentacular nerve. til, left inferior tentacular artery. tir, right inferior tentacular artery. tl, left tentacular artery. tr, right tentacular artery.

21

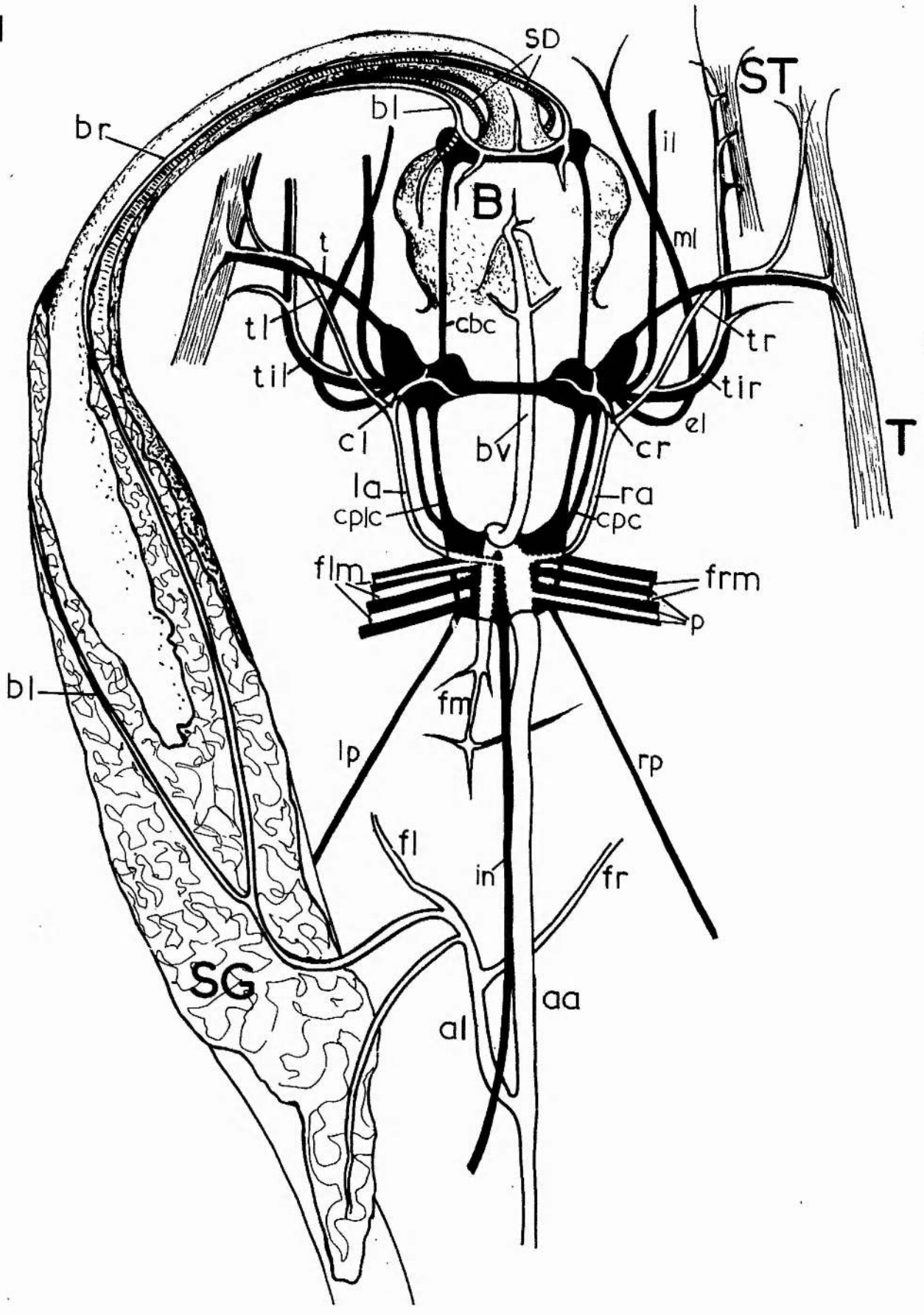
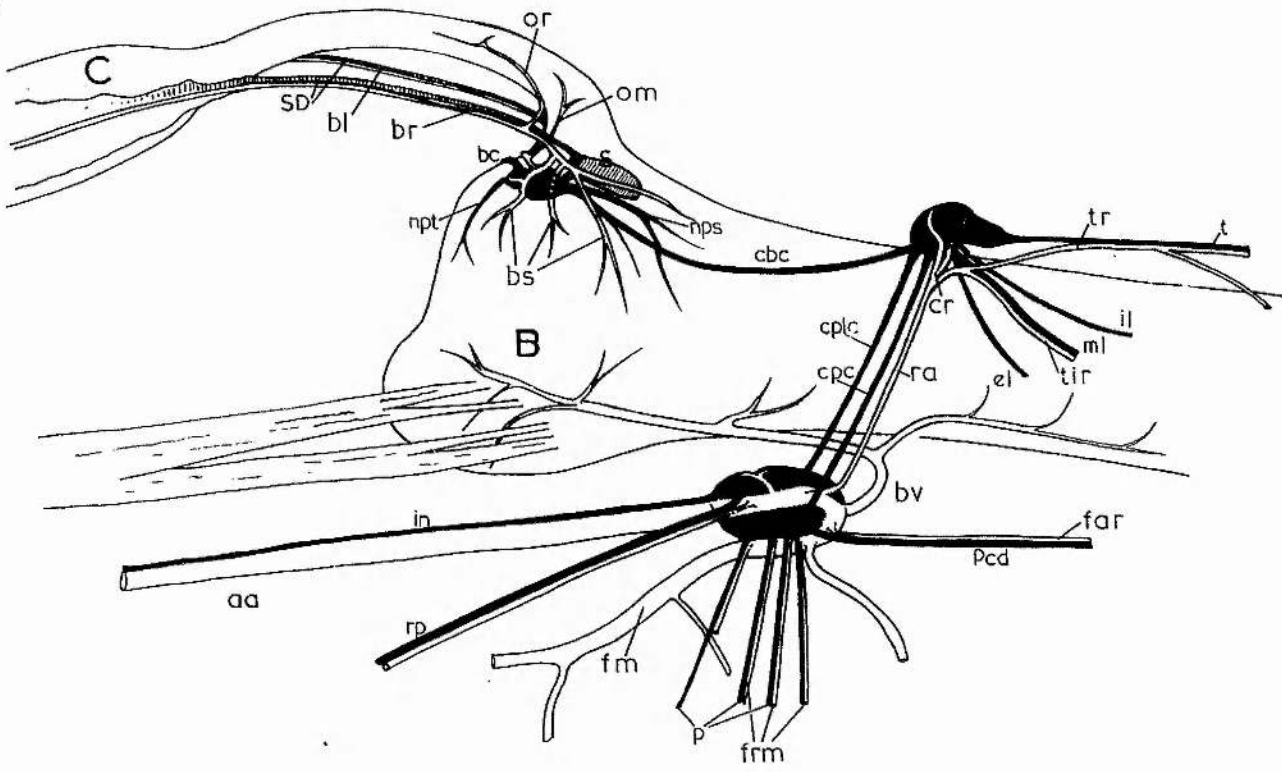

SD
ST
bl
br
il
B
ml
t
tl
cbc
tr
til
tir
el
cl
bv
cr
T
la
ra
cplc
cpc
flm
frm
p
bl
fm
lp
rp
fl
in
fr
SG
aa
al

Fig. 22. Diagram showing the general blood supply to the central ganglia of Helix pomatia in lateral view. (In this diagram the buccal mass (B) is in the retracted position). B, buccal mass. C, crop. S, salivary duct opening. SD, salivary duct. aa, anterior aorta. bc, buccal commissure. bl, left buccal artery. br, right buccal artery. bs, dorsal buccal artery(s). bv, ventral buccal artery. cbc, cerebro-buccal connective. cpc, cerebro--pedal connective. cplc, cerebro-pleural connective. cr, right cerebral artery. el, external lip nerve. far, right anterior foot artery. fm, middle foot artery. frm, right lateral foot artery(s). il, internal lip nerve. in, intestinal nerve. ml, median lip nerve. nps, second pharyngeal nerve. npt, third pharyngeal nerve. om, medial oesophageal artery. or, right oesophageal artery. p, pedal nerve(s). pcd, right cutaneous foot nerve. ra, right anterior artery. rp, right pallial nerve. t, tentacular nerve. tir, right inferior tentacular artery. tr, right tentacular artery.

Fig. 23. Dorsal view of the blood supply to the cerebral ganglia of Helix pomatia. The connective tissue is not shown. The anterior arteries (ra, la) may give rise to one (cr) or two (cl) branches which ramify over the surfaces of the ganglia. The left cerebral ganglion has been cut away to illustrate the final blood space that lies very close to the nervous tissue. Capillaries do not penetrate the nervous tissue. Each anterior artery gives a further branch (tir, til) which follows closely the median lip nerves (mlr) and eventually supplies the small tentacle. The anterior arteries leave the tentacular arteries (tl, tr) which follow the tentacular nerves to supply the large tentacles, and on the right side the penis as well. M, mesocerebrum. Mt, metacerebrum. P, procerebrum. cbc, cerebro-buccal connective. cc, cerebral commissure. cl, left cerebral artery(s). cpc, cerebro-pedal connective. cplc, cerebro-pleural connective. cr, right cerebral artery(s). elr, right external lip nerve. ilr, left internal lip nerve. la, left anterior artery. mlr, right median lip nerve. nacr, right anterior artery nerve. npir, right internal peri-tentacular nerve. ra, right anterior artery. til, left inferior tentacular artery. tir, right inferior tentacular artery. tl, left tentacular artery. tnl, left tentacular nerve. tnr, right tentacular nerve. tr, right tentacular artery.

22


C
or
om
SD
bl
br
bc
npt
nps
bs
cbc
tr
cr
il
ml
tir
cplc
cpc
ra
el
B
bv
in
far
Pcd
aa
rp
fm
p
frm

23

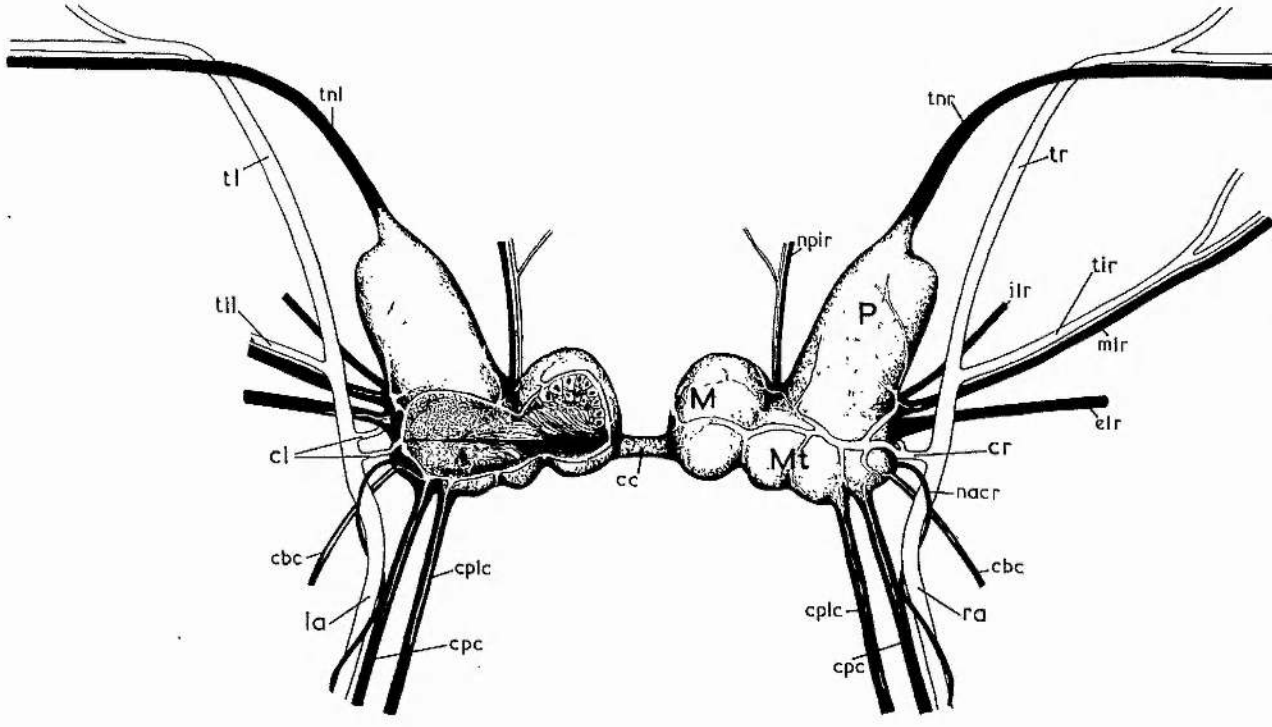

tnl
tl
til
cl
cbc
la
cplc
cpc
cc
M
Mt
P
npir
tnr
tr
tir
jlr
mlr
elr
cr
nacr
cbc
cplc
ra
cpc

Fig. 24. Diagram showing a dorsal view of the blood supply to the sub--oesophageal ganglionic complex (connective tissue removed) of Helix pomatia. The left parietal and left pleural ganglia have been cut away to show the vessels supplying the pedal ganglia. The anterior aorta curves anteriorly between the pedal ganglia, eventually turning posteriorly to serve the foot. Small vessels leave the aorta and its principal branches. These penetrate the connective tissue sheath to form an almost continuous blood space covering the nervous tissue, which is indicated in the cut away ganglia in the middle and left. Capillaries accompany each nerve which leaves the various ganglia. F, foot. P, left pedal ganglion. Pl right pleural ganglion. Pr, right parietal ganglion. V, visceral ganglion. aa,anterior aorta. aan, anterior aorta nerve. an, anal nerve. bv, ventral buccal artery. cpc, cerebro-pedal connective. cplc, cerebro-pleural connective. fm, middle foot artery. fn, foot nerves with associated arteries. in, intestinal nerve. la, left anterior artery. laf, left anterior foot artery. nmc, columnella muscle nerve. nrp, pharyngeal retractor muscle nerve. pc1, first left cutaneous pedal nerve. pc2, second left cutaneous pedal nerve. pc3, third left cutaneous pedal nerve. ppc, pleuro-pedal connective. ra,right anterior artery. raf, right anterior foot artery. rpn, right pallial nerve.

24

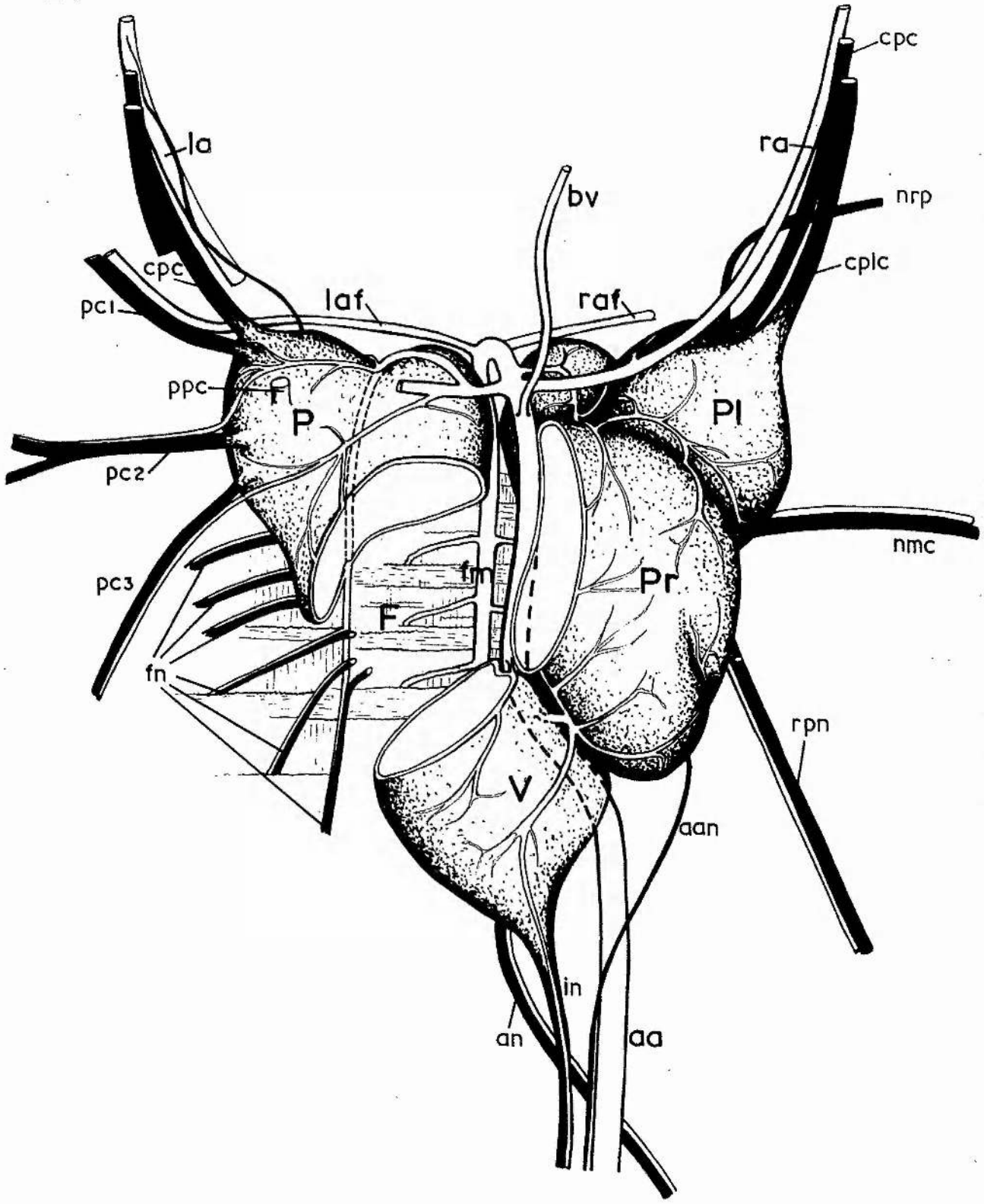
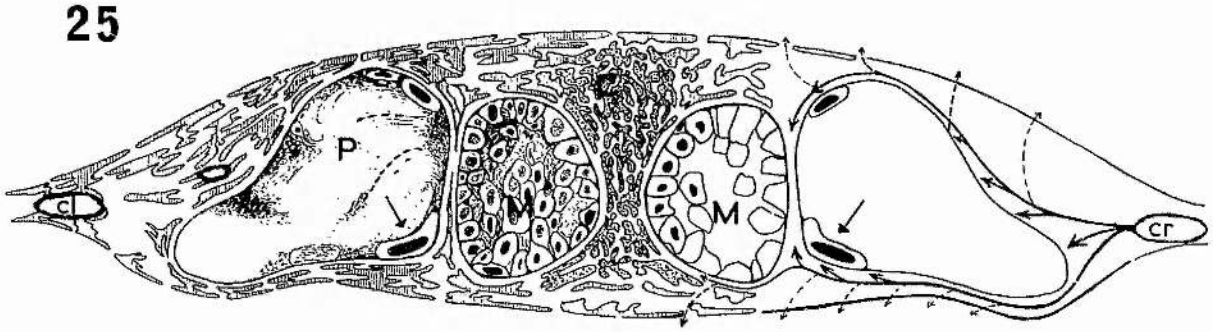

cpc
la
ra
bv
nrp
cplc
cpc
laf
raf
pc1
ppc
P
Pl
pc2
nmc
pc3
fm
Pr
F
fn
rpn
V
aan
in
an
aa

Fig. 25. Diagram of a vertical section through the mid region of the cerebral ganglia and connective tissue sheath of Helix pomatia. The left half of the section is accurately reproduced from an ink-injected preparation. The right half of the section shows the way in which blood passes over the nervous tissue, and through the connective tissue sheath. It is apparent that the GSC at the ventral edge of the metacerebrum, Mt (arrow), receives a good blood supply because it has a large part of its surface lying very close to the blood spaces. cl, left cerebral artery. cr, right cerebral artery. M, mesocerebrum, Mt, metacerebrum.

Fig. 26. Diagram showing the blood supply to the buccal ganglia of Helix pomatia in posterior view. The superficial musculature and connective tissue sheath have been removed and the buccal mass is in the retracted position. Each buccal artery (bl, br) branches several times before reaching the ganglia. A portion of the right buccal ganglia is cut away to show how several vessels pass over the surface of the ganglion, leaving a blood filled space which covers the nervous tissue. Blood passes from this space through the connective tissue into the body sinuses. We have adopted the nomenclature of Schmalz (1914) for the buccal nerves. B, buccal mass. D, opening of salivary duct. SDl left salivary duct. SDr, right salivary duct. bl, left buccal artery. br, right buccal artery. cbc, cerebro-buccal connective. npl1, first left pharyngeal nerve. npl2, second left pharyngeal nerve. npl3, third left pharyngeal nerve. nprl, first right pharyngeal nerve. npr2, second right pharyngeal nerve. npr3, third right pharyngeal nerve. oal, left anterior oesophageal nerve. oar, right anterior oesophageal nerve. ol, left oesophageal artery. om, medial oesophageal artery. opl, left posterior oesophageal nerve. opr, right posterior oesophageal nerve. or, right oesophageal artery. sgl, left salivary gland nerve. sgr, right salivary gland nerve.

25


P
M
M
cr

26

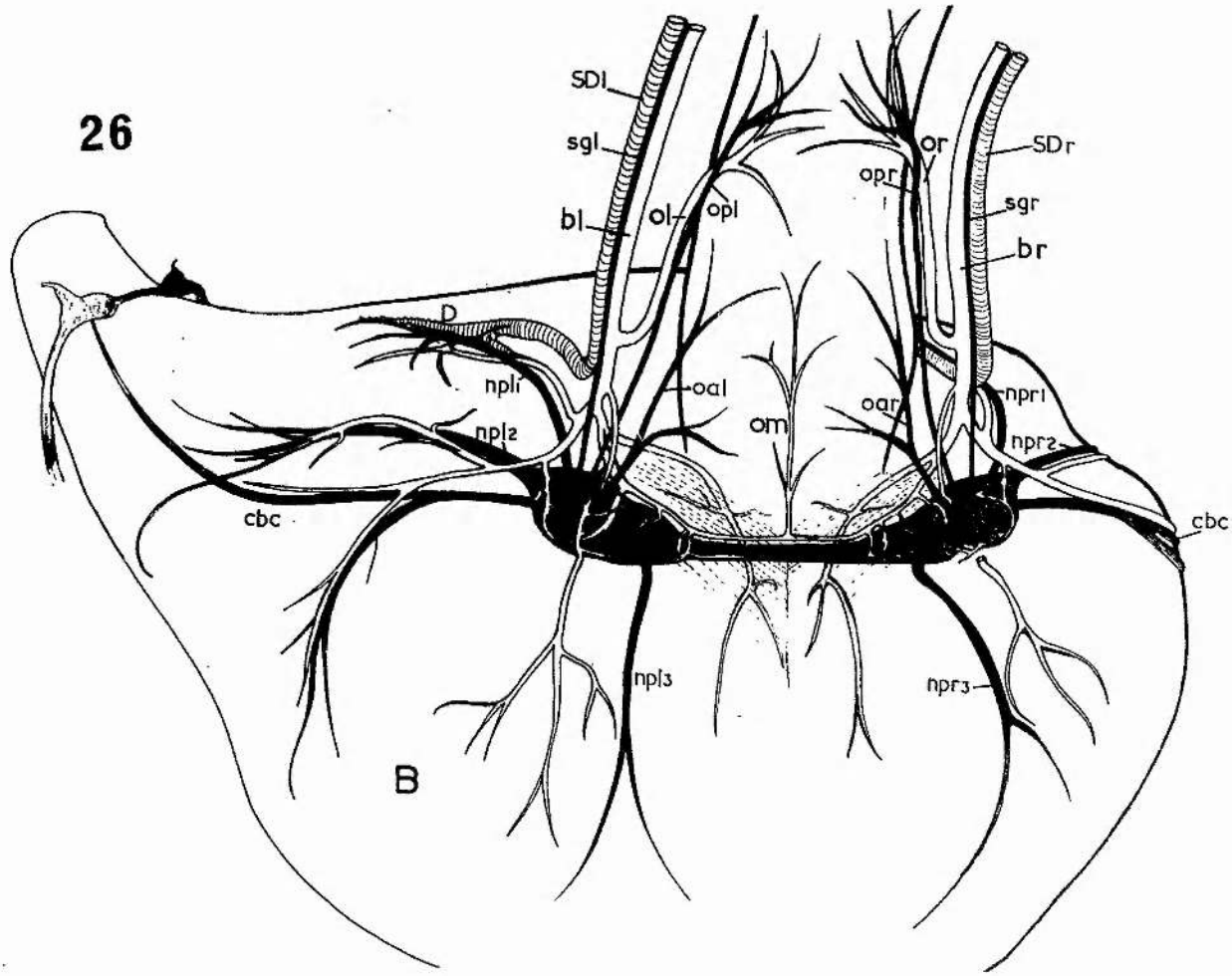
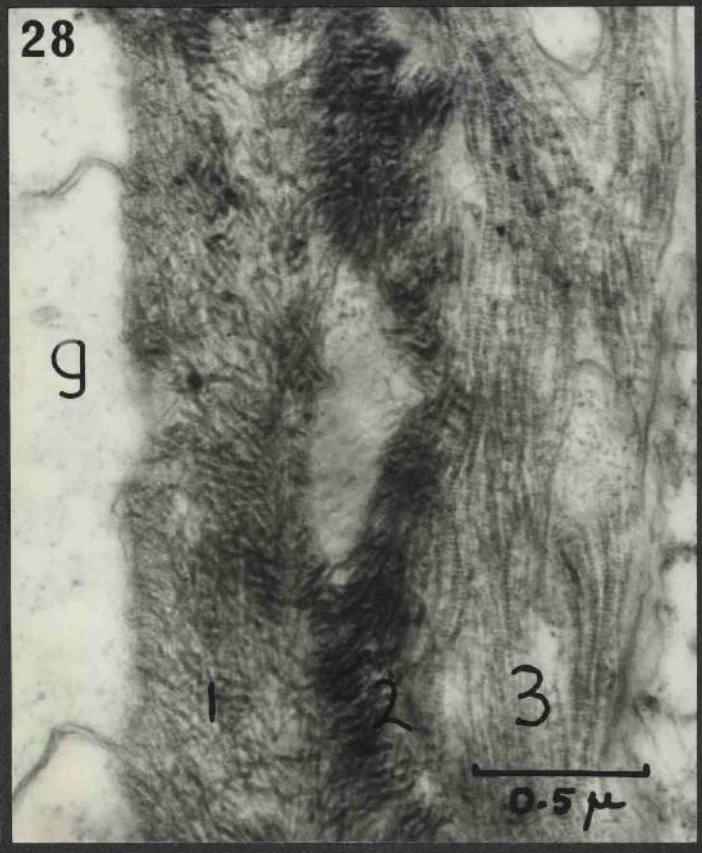

SDl
sgl
bl
ol
opl
P
npli
npl2
oal
om
cbc
npl3
B
or
opr
SDr
sgr
br
oar
npr1
npr2
cbc
npr3

Fig. 27. Electron micrograph of a small capillary in the connective sheath of the cerebral ganglion of Helix pomatia. The endothelial cell (nucleus, N) produces a folded wall (en) around the vessel. Collagen (cl) surrounds the vessel closely. L, lumen of vessel. The cell marked G appears very similar to the globular cells described by Rogers (1969) in the epineural sheath of Helix aspersa.

Fig. 28. Electron micrograph showing the connective tissue layer covering a part of the procerebrum of Helix pomatia. The collagen strands appear to be in three concentrated layers (1-3). 1 and 2 run perpendicularly to each other but obliquely with respect to the glial cells (g), while 3 is seen in longitudinal section and runs parallel to the glial cells.

Fig. 29. Electron micrograph of a part of the connective tissue sheath covering the sub-oesophageal ganglia of Helix pomatia. In the mid-dorsal region (shown here) the bulk of the sheath is composed of collagen fibres (C). In this section the majority of these fibres are cut in cross-section, but a small number are cut obliquely (arrows). Several muscle cells (M) are also seen in cross-section. N, nucleus of muscle cell.


27
G
N
L
en
cl
μ


28
g
1
2
3
0.5 μ

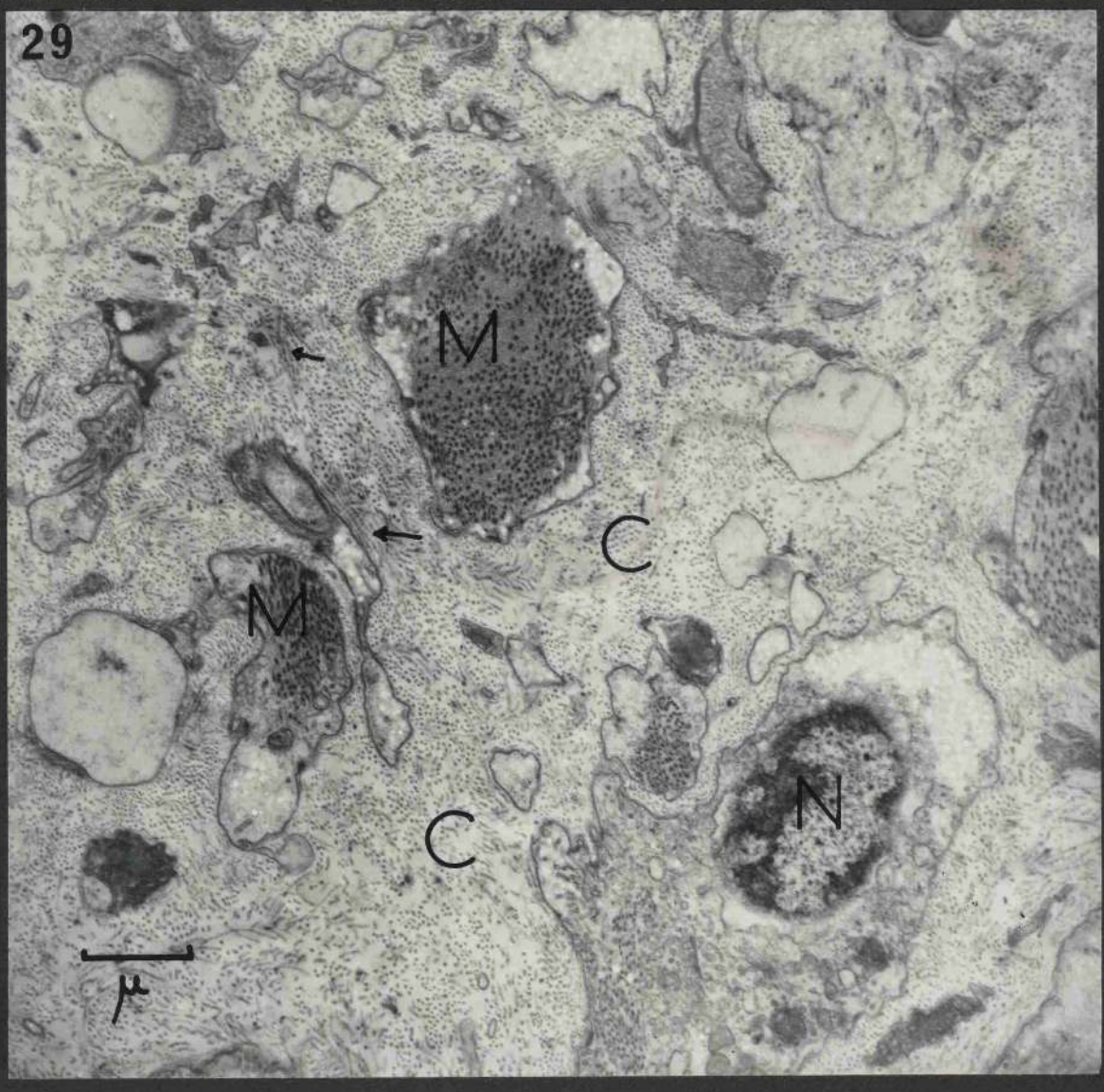

29
M
M
C
C
N
μ

Fig. 30. The blood spaces (bs) surrounding the cerebral ganglia are bounded by elongated endothelial cells (en). These cells sometimes cross the blood spaces, forming a supporting network between the sheath and the nerve tissue. The section shows part of the dorso-lateral edge of a GSC. Here there are many collagen fibres in the connective layer (cl). These fibres are not so plentiful on the outermost ventral edge of the cell (cf. Fig. 34). A glial cell (G) sends finger-like processes (f) into the cell's cytoplasm. N, nucleus of endothelial cell. g, Golgi body. x, lysosome-like bodies.

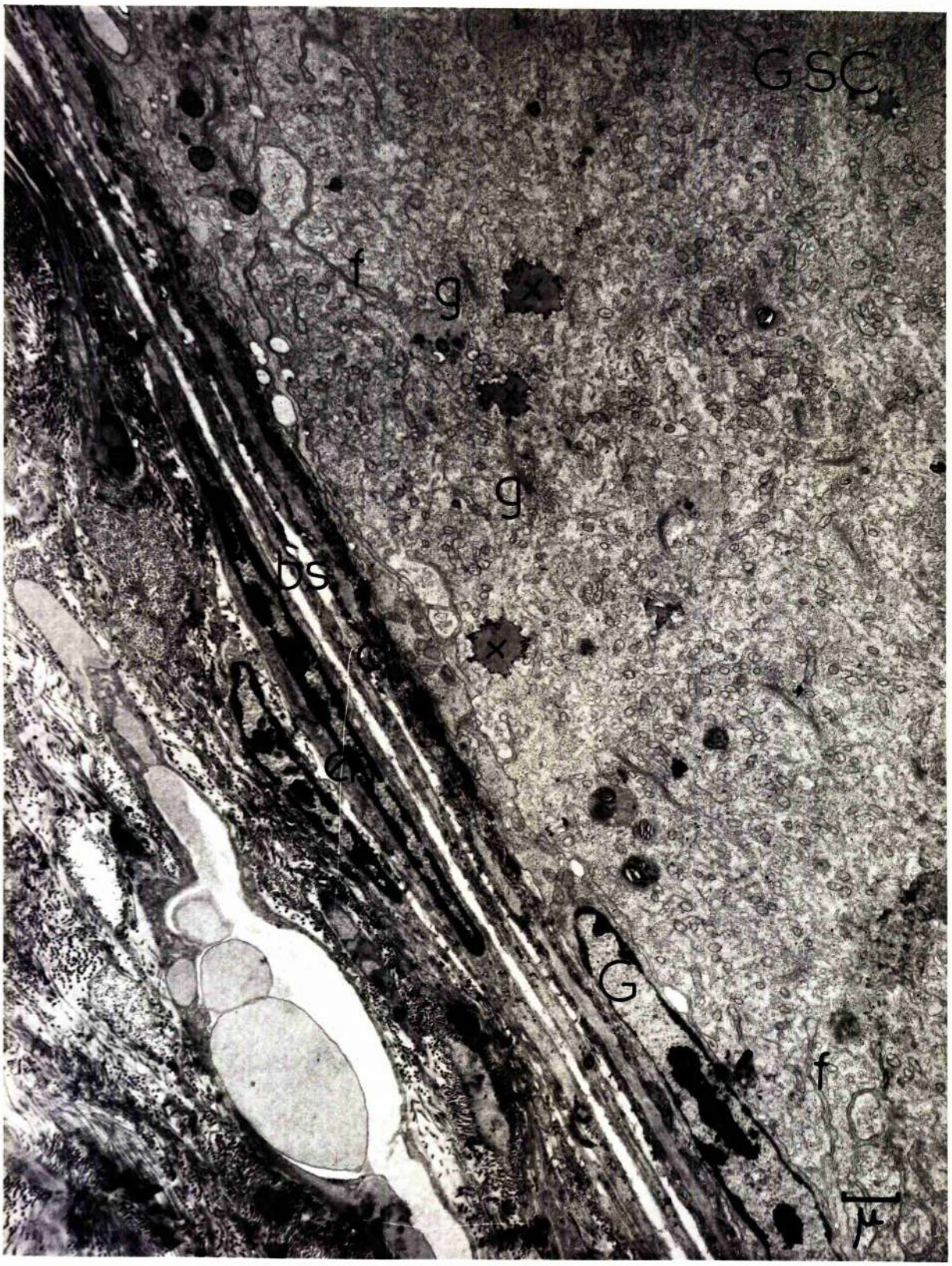
Fig. 31. Electron micrograph of a part of the connective sheath covering the visceral ganglion of Helix pomatia. A small nerve, cut obliquely, is composed of several axons which contain either clear vesicles (mean diameter 50 nm) (CV), or dense-cored vesicles (mean diameter 120 nm). C, collagen. M, muscle cell. The micrograph illustrates the apparently complex and twisted arrangement of the collagen in contracted sheath tissue.

Fig. 32. Electron micrograph of a part of the connective tissue sheath covering a cerebral ganglion of Helix pomatia. The micrograph shows an axon, cut in cross-section, which contains dense-cored vesicles of mean diameter 120 nm. Many axons similar to this are present throughout the sheaths covering the sub-oesophageal ganglia and peripheral nerves of Helix pomatia. The significance of such axons, and of those containing clear vesicles (see Fig. 31) is not clear. It is possible that some of them may synapse onto muscles within the sheath (see Benjamin and Peat, 1968). Alternatively, some of these axons may have a neurosecretory function. In relation to this Fernandes and Fernandes (1972) have classified axons in the ganglionic sheaths of Helix aspersa as either 'aminergic or peptidergic' on the basis of their vesicle content. The 'peptidergic' axons figured by these authors contain vesicles of similar electron opacity and diameter to those shown in this micrograph. Because some such 'peptidergic' axons in Helix aspersa were seen to end apparently blindly within the connective tissue, Fernandes and Fernandes (1972) postulated that in this animal the sheath could be considered a neurohaemal organ.

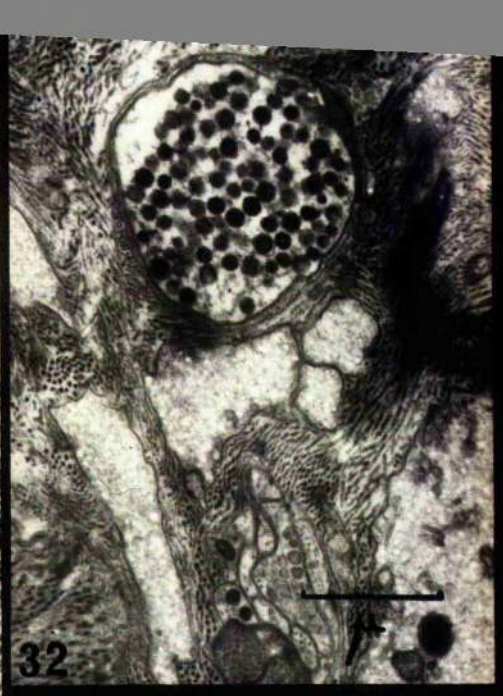
Fig. 33. Electron micrograph of a part of a cerebral ganglion (lateral mesocerebrum) of Helix pomatia which had been injected with ink and left for 24 hr. The blood spaces (B) are separated from a glial cell (G) by a thin endothelium (EN) and a thick layer of connective tissue. The collagen in this layer (cl) is not tightly packed, and contains several spherical electron-dense bodies. C, cytoplasm of neuron.

Fig. 34. Electron micrograph of section through the same tissue as that in Fig. 33, but taken at the outer ventral edge of a GSC (cf. Fig. 25). Here the blood space (colloidal carbon) is expanded. A continuous layer of luminal endothelium (en), and a relatively thin layer of connective tissue (cl), separate the glial cell membranes (g) from the blood space. C, cytoplasm of GSC.

30




C
SV
M
31


32


En
Cl
g
C
34

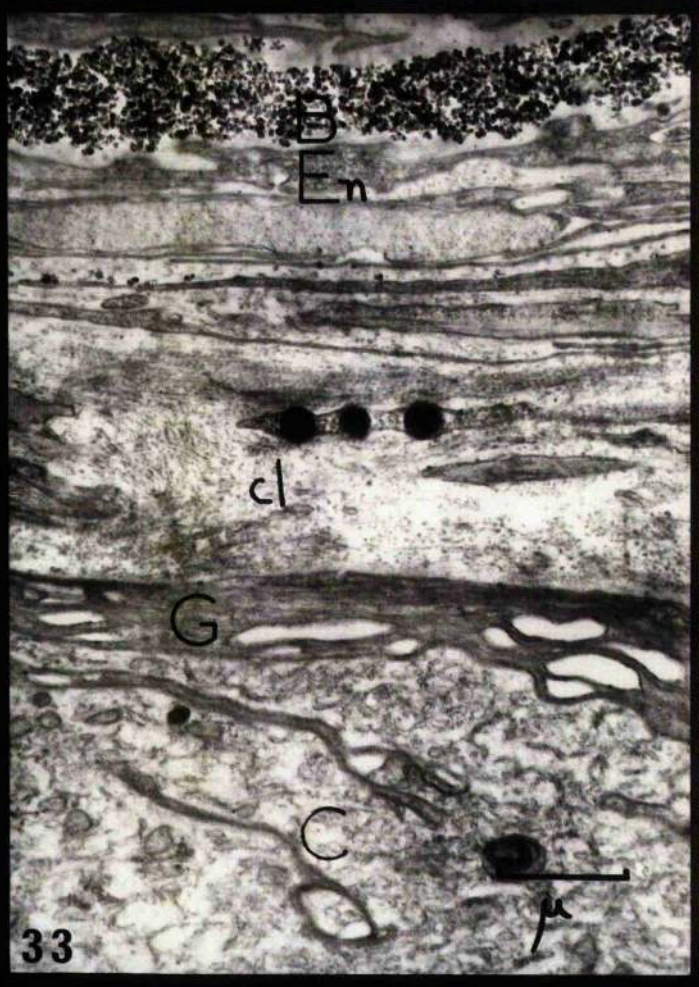
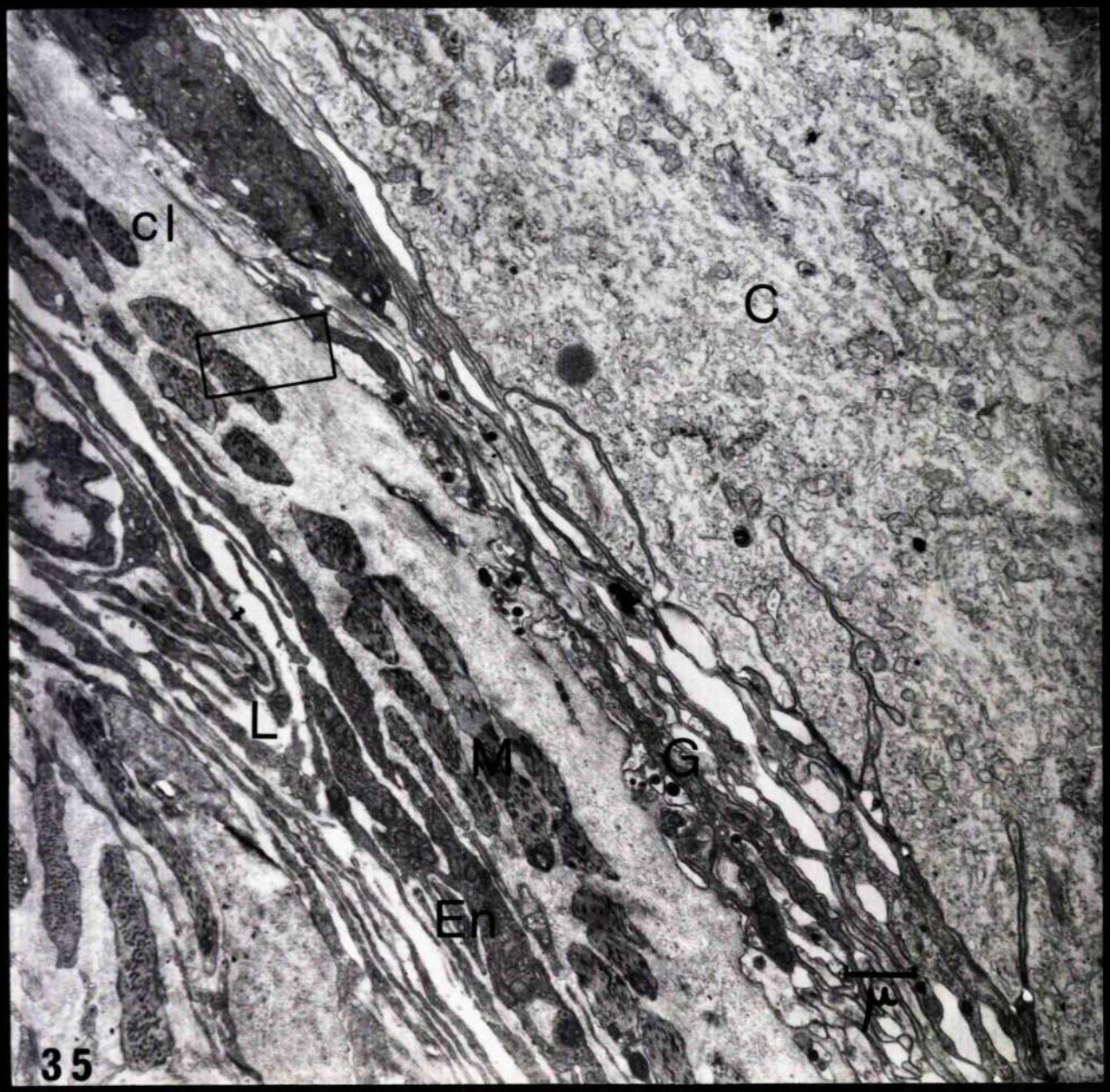

B
En
cl
G
C
33

Fig. 35. Electron micrograph of section through the edge of the metacerebrum of an aminal which had been injected 20 min previously with ferritin. At this magnification the tracer cannot be seen. The inset is shown in Fig. 36. L, lumen of blood spaces. EN, endothelium. M, muscle cells in the connective tissue (cl). G, glial cell processes. C, cytoplasm of neuron.

Fig. 36. At high magnification (see inset of Fig. 35) it is evident that ferritin particles have diffused to the glial cell membranes (G). They do not appear to have penetrated between the extracellular glial spaces (arrows). M, muscle cell. Cl, collagen.


cl
C
L
M
G
En
μ
35

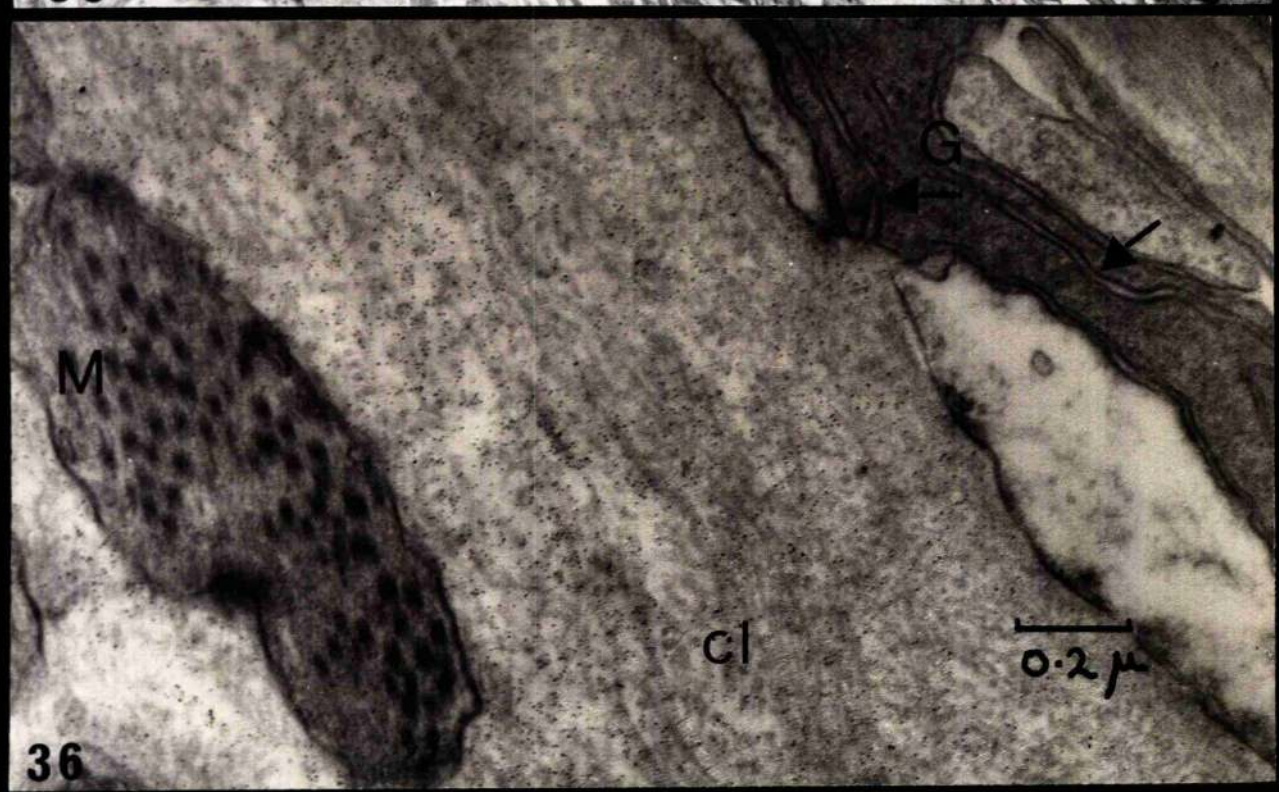
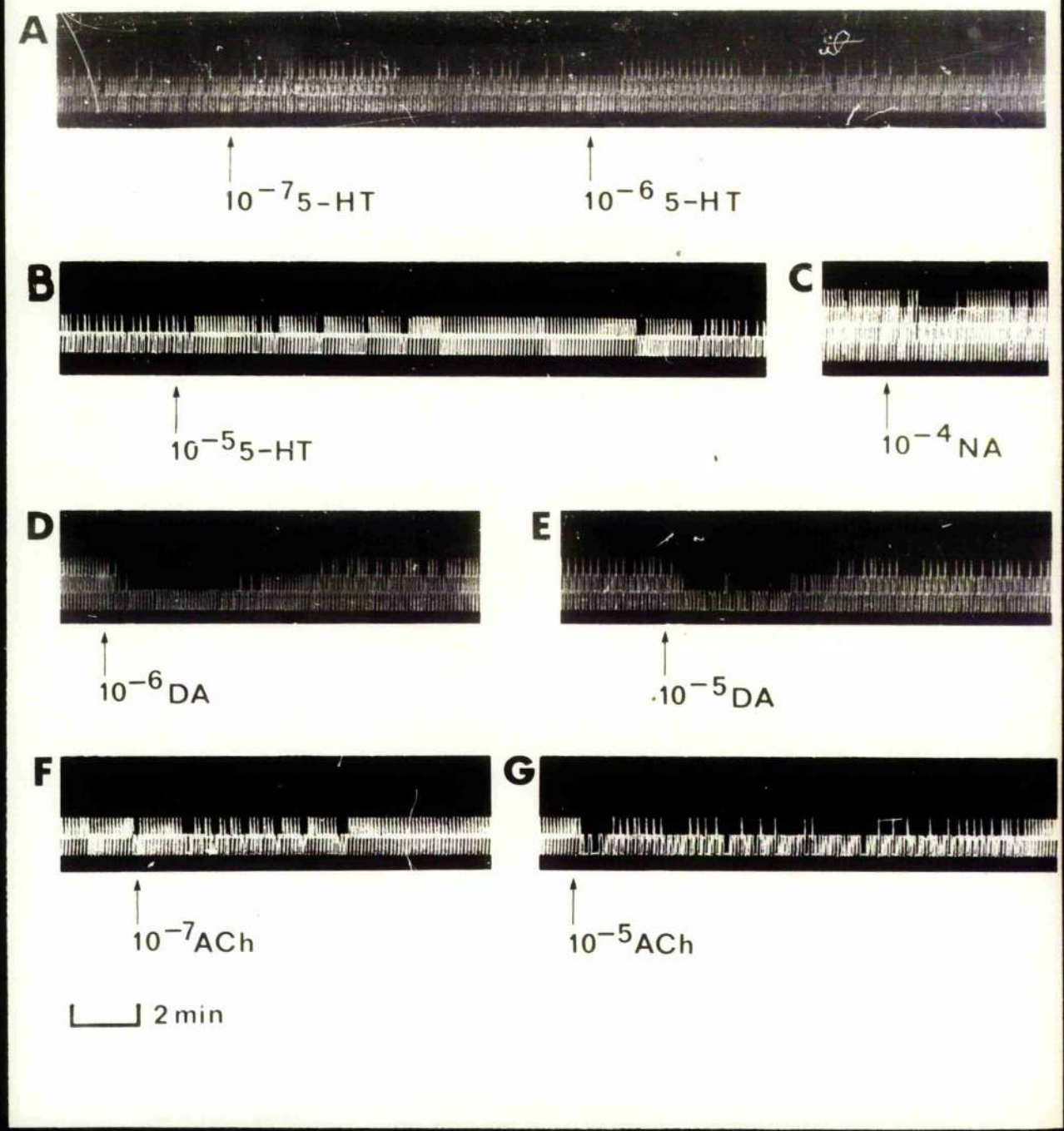

G
M
cl
0·2μ
36

Fig. 37. The effect of perfusing the isolated anterior aorta and cerebral ganglia with amines and acetylcholine. Each stepped 'spike' in the tracings is one drop recorded by a phototransistor. In (A) 5-HT (10^{-7}, 10^{-6} g/ml in the perfusing medium) increases the rate of flow through the aorta by approximately 10%. (B) At a concentration of 10^{-5} 5-HT g/ml perfusing saline the flow rate through the cerebral ganglia is increased by approximately 30%. C, D and E show the restricting effect of noradrenaline (NA) and dopamine (DA) on the perfused aorta. These drugs produced a similar effect on the rate of flow through the isolated cerebral ganglia. F and G show the graded reduction in flow through the cerebral ganglia caused by increased concentrations of acetylcholine.

37


A
10^{-7} 5-HT
10^{-6} 5-HT
B
10^{-5} 5-HT
C
10^{-4} NA
D
10^{-6} DA
E
10^{-5} DA
F
10^{-7} ACh
G
10^{-5} ACh
2 min

Chapter 3

SELECTIVE UPTAKE OF 5-HT BY AXONAL PROCESSES IN *Helix pomatia*

Introduction

Various mechanisms have been proposed for terminating the actions of neurotransmitter substances released from presynaptic nerve terminals. They include (1) metabolic conversion to inactive substances, (2) diffusion away from the synaptic area, and (3) cellular uptake from the region of the synaptic cleft; for example, re-uptake into the presynaptic terminals.

Perhaps the most familiar mechanism is the metabolic inactivation of acetylcholine by the enzyme acetylcholinesterase at cholinergic synapses. However, recent data suggests that this situation may, in fact, be exceptional. On the other hand, there is growing evidence that, in mammals, a re-uptake mechanism is chiefly responsible for inactivating noradrenaline, and probably also the proposed transmitters 5-HT, dopamine, GABA and glycine (see Iversen, 1971).

If 5-HT serves a transmitter role in molluscs, an important aspect of this role would appear to be the mechanism of its inactivation in extracellular spaces, after its presumed release at synapses. Several authors (i.e. Kerkut and Cottrell, 1963; Cardot, 1963, 1964; Juorio and Killick, 1972b) have reported that MAO does not appear to inactivate 5-HT in *Helix* ganglia (see introduction). Thus it would appear that this enzyme is not significantly involved in inactivating 5-HT in the CNS of this animal. On the other hand, Gerschenfeld and Stefani (1968) have provided some evidence that diffusion may play a role in reversing the effect of 5-HT on some specified neurons of *Cryptomphallus aspersa*.

Alternatively, a cellular uptake of 5-HT may be involved. The evidence in favour is as follows: (1) The antidepressant drug imipramine, which blocks

5-HT uptake by mammalian blood platelets and central neurons (Da Prada and Pletscher, 1968; Carlsson, Fuxe and Ungerstedt, 1968), blocks the uptake of 5-HT by the central ganglia of H. pomatia (Cottrell, 1971b), and also markedly potentiates transmission between the GSC and buccal ganglion cells in this animal (Cottrell, 1971c). (2) Auricles of Aplysia accumulate 5-^{3}HT to a concentration which is approximately tenfold that of the bathing medium (Chase, Breese, Schanberg and Kopin, 1971). This accumulation is inhibited by desmethylimipramine, ouabain and Na^{+}-free solutions, which indicates that it is an active transport process (Carpenter, et al., 1971). Furthermore, Carpenter et al. (1971) found a close relationship between the content of endogenous 5-HT in auricle and ventricle, and their ability to concentrate 5-^{3}HT. Some of the labelled 5-HT accumulated by the Aplysia heart can be released by electrical field stimulation of the heart, or stimulation of the cardiac nerves (Chase et al., 1968). Taxi and Gautron (1969) have demonstrated 5-HT specific fluorescent varicosities in the heart of Aplysia. (3) Taxi and Gautron (1969) have also shown, by E.M. autoradiography, that 5-HT is taken up into nerve endings in the Aplysia heart.

At variance with those data, attempts to show active uptake of 5-HT by central ganglia of molluscs have so far failed. Aplysia ganglia incubated in vitro with 5-^{3}HT will not accumulate it more than three times the external concentration (Chase et al., 1968; Carpenter et al., 1971). Autoradiographic experiments by Ascher, Glowinski, Tauc and Taxi (1968) have shown that much of this 5-HT is accumulated in the connective tissue sheath. Ascher et al. (1971) suggested that uptake of 5-^{3}HT by central ganglia of Aplysia was non-specific (see also Gerschenfeld, 1973).

However, the work of Chase et al. (1968) and Ascher et al. (1968) can be criticised because of the experimental procedures employed. First, these workers exposed ganglia to high concentrations of 5-HT. Iversen (1971) has shown that exposure of mammalian nervous tissue to high concentration of noradrenaline (greater than 1 µg/ml perfusing medium) results in a non-specific

uptake of this substance ($Uptake_2$), which is different from the selective uptake of low concentrations (less than 0.5 µg/ml perfusing medium) of this substance by noradrenergic nerve endings ($Uptake_1$). Although $Uptake_1$ occurs with both high and low concentrations of noradrenaline in the perfusing medium, $Uptake_2$ masks $Uptake_1$ at high concentrations. If a similar situation should exist with the uptake of 5-HT, then clearly the results of Ascher et al. (1968) reflect a non-specific uptake of this amine in Aplysia ganglia, with consequent masking of any selective uptake by nerve endings. Second, these workers exposed isolated Aplysia ganglia to 5-^{3}HT. The previous chapter, however, has shown fairly clearly that in Helix pomatia, blood is in vivo channelled directly to the surface of the nervous tissue, and then passes out through the connective tissue. If a similar arrangement exists in Aplysia, which is likely (chapter 2), then clearly 5-HT would have easier access to nerve endings in the central nervous tissue if it were perfused via the blood system.

In the present chapter, the site(s) of 5-HT uptake in the nervous system of H. pomatia is investigated. Autoradiographic techniques are employed. An attempt is made to avoid the difficulties described above by (1) employing low concentrations of 5-HT, and (2) by perfusing the intact arterial system of the animal. Furthermore, the uptake of 5-HT is studied especially with respect to areas of the animal thought to contain presynaptic endings of the GSC (i.e. buccal ganglia neuropile and lip muscles).

Materials and Methods

Only active specimens of Helix pomatia were used experimentally.

Uniformly tritium labelled 5-HT creatinine sulphate (12.0 Ci/m mol) was obtained from the Radiochemical Centre, Amersham, England. Radioactive contaminants as measured by paper chromatography were less than 3%.

(1) Perfusion of the intact nervous system

To obtain as near as possible normal conditions, the brain was perfused via the anterior aorta with saline (Meng, 1960) containing 0.5-1.5 nM labelled 5-HT. Test solutions were perfused through the brain for 1-16 hr at rates of

2-4 ml/hr at 16 to 20°C. The brains were then perfused for a further 30 min with saline alone.

(2) Experiments with the isolated central nervous system

The central ganglia were removed and pinned out in small dishes containing saline. The outer connective tissue sheath was removed by dissection and 5-^{3}HT was added to give a bath concentration of 1.0-1.5 nM. Preparations were left for 1-10 hr at 18°C, and subsequently washed in a flow of saline for 30 min.

(3) Autoradiographic procedures

After exposure to labelled 5-HT the central ganglia, in some cases together with peripheral nerves and musculature, were fixed by perfusion and/or immersion in 2.5% gluteraldehyde in 0.3 M cacodylate buffer (pH 7.4) for 2-4 hr, and then post-fixed in 1% $O_s O_4$ in 0.2 M cacodylate buffer for 1½ hr.

For light microscope autoradiography, tissues were dehydrated (ethanol series), cleared (xylene) and embedded in paraffin wax. 10 μ serial sections were mounted on gelatin-coated slides, and covered with Kodak AR.10 stripping film. The film was exposed for 2 days to 2 weeks and subsequently developed in Kodak D.19 developer. In some cases, sections were stained with 1% Toluidine Blue; more frequently staining was omitted because sufficient contrast was present in tissues post-fixed in osmic acid.

For electron microscope autoradiography, tissue was dehydrated in a series of acetone-water solutions and embedded in Araldite. Pale gold sections were mounted on 300 mesh copper grids, stained with lead citrate and uranyl acetate, and coated with 2-5 nm of evaporated carbon. The grids were placed on glass slides and coated with Ilford L.4 emulsion (diluted 1:1 with distilled water and liquefied by heating to 40°C for 30 min) carried in a loop of silver wire (Caro, Tubergen and Kolb, 1962). After 3-10 weeks exposure, grids were developed in Kodak Nikrodol-K for 5 min at 22°C, fixed in acid fixer (Kodak) and examined in the electron microscope. For comparison, 1 μ Araldite sections were placed on gelatin-coated slides, coated with 2-5 nm of carbon, and processed for light microscopy with Kodak AR.10 as described above.

4 light microscope experiments and 3 electron microscope experiments were made with intact (perfused) preparations. 3 light microscope experiments were made with the isolated central ganglia.

(4) Chemical procedures

The amounts of radioactive 5-HT retained in the various ganglia and in single neurons after washing with saline were estimated by liquid scintillation. Individual neurons were dissected by hand with fine-tipped forceps and tungsten needles. Tissues were placed in vials containing a tissue solubiliser (NE520, Nuclear Enterprises Ltd., Glasgow), and counted in a Packard Model 3320 Scintillation Spectrometer.

Results

Under the experimental conditions used, 5-HT was taken up selectively by some axonal processes in the neuropile of each ganglion, and others in the connective tissue sheaths of each ganglion, and in peripheral musculature (e.g. the lip muscles). The results appeared very similar for both the in vivo and in vitro experiments. Furthermore, a similar distribution of labelled axons was found in both the experiments at the light microscope level, which employed serial sections, and the experiments at the electron microscope level.

Figs. 38, 40 show sections through the buccal ganglion. It is apparent that labelled structures are chiefly present in neuropile regions and in the connective tissue sheath (Fig. 39). Some of the labelled structures appear to be axon processes. The distribution of label suggests, furthermore, that some such axon processes are varicose in nature. Some of the labelled structures are in close contact with the proximal axon branches of a giant buccal cell which receives synaptic connection from each GSC (Cottrell, 1971a).

Figs. 42-46 are electron microscope autoradiographs showing labelled structures in the neuropile of the cerebral and buccal ganglia. These micrographs illustrate clearly that 5-^{3}HT does in fact label axonal processes. They also show that such labelled processes contain dense-cored vesicles of mean diameter 100 nm (see Figs. 42, 44, 45, 46, insets). These vesicles are morpho-

logically very similar to those thought to sequester 5-HT in the GSC perikarya (see chapter 1). However, it should be noted that the autoradiographic technique does not have sufficient resolution to show whether or not such vesicles are the actual loci of radioactivity within the nerve endings.

The GSC sends one or more axon branches into the external lip nerve (Kandel and Tauc, 1966a). Evidence is presented in chapter 5 that such axons have their endings on, or very near, muscles in the mouth of the animal. The external lip nerve contains approximately 10^5 parallel-running axons (Fig. 66), but only twenty or so of these were seen in any cross-section of the nerve to be significantly labelled (Fig. 50). A small proportion of axons on the lip muscles also took up labelled 5-HT. Such axons often showed a varicose appearance (Fig. 41), and appeared to be closely associated with muscle cells (Fig. 47).

Certain structures in the connective tissue sheath of each ganglion (Figs. 38, 39) accumulate 5-^{3}HT. These again often exhibited a varicose appearance (Fig. 39), and appeared to be axons or nerve endings (Fig. 48) some of which contained dense-cored vesicles (Fig. 49).

No evidence was obtained that any muscle cell, glial cell, or unexpectedly, any nerve cell body (5-HT containing, eg. i.e. the GSC, or otherwise) was significantly labelled after exposure to labelled 5-HT.

The experiments to determine the absolute amounts of radioactive 5-HT retained in the various ganglia and in single neurons add support to the autoradiographic findings: The entire brain contained an average of 0.1 µg of labelled 5-HT after exposure to this substance, whereas both GSCs and giant buccal neurons contained radioactive counts which did not exceed the background count. (However, in one experiment out of a total of six--six cells of each type were used per experiment--the GSC was found to contain 1.2 n Ci, equivalent to 80 pg, of 5-^{3}HT; whereas the buccal cells contained only a background level. This exceptional result may have been due to contamination, but it is included because it may indicate that under occasional conditions,

which are not at present understood, the GSC may in fact take up 5-HT).

Discussion

The results presented above show that a small proportion of axons in central and peripheral nervous tissue of Helix pomatia are selectively labelled after exposure to 5-^{3}HT in vivo and in vitro. Some of these labelled axons are present in areas thought to contain presynaptic endings of the GSC (i.e. in the buccal ganglia and muscles in the mouth of the animal). It is possible that some, if not all, of the labelled axons are nerve endings, because (1) such structures are often varicose (light microscope results), and (2) they contain large numbers of vesicles (electron microscope results). Some of the vesicles present in the labelled axon processes contain dense cores, and are morphologically similar to those present in the GSC perikaryon.

However, autoradiographic studies have several limitations. For example, they provide no data as to whether or not the axonal processes are labelled as a result of an active uptake process, i.e. it is not possible to say whether the labelled processes contain a greater concentration of 5-^{3}HT than that present in the perfusing medium. It would be possible to clarify this point by making autoradiographic experiments of ganglia exposed to labelled 5-HT in the presence of ouabain or Na^{+}-free solutions. However Cottrell (1971b) has shown that imipramine (concentration 3.5×10^{-5} M) blocks by about 80% the uptake of 5-HT by the brain of H. pomatia, which suggests that an active process may be involved (see also chapter 4).

Another important factor is that the autoradiographic procedures probably only allow visualization of bound 5-HT. The process of fixation, dehydration and coating with emulsion, will presumably wash out all unbound, freely soluble 5-HT. No experiments were made to determine the extent to which this happened in the present work. However, Gerschon and Ross (1966), who studied the uptake and binding of 5-^{3}HT synthesized from administered 5-^{3}HTP in the parafollicular cells of the mouse, found that essentially all unbound 5-HT was washed out prior to autoradiography. The experimental procedure (i.e.

gluteraldehyde and osmium fixation, and dehydration in ethanol) employed in the present work was similar to that used by Gerschon and Ross (1966), hence it is likely that the present autoradiograms represent bound 5-HT. It would be perhaps possible to test this by studying freeze dried tissues autoradiographically, without conventional fixation, after exposure to 5-^{3}HT.

A closely related problem is the relatively long time periods (3-15 hr) of exposure to labelled 5-HT necessary to produce clear-cut autoradiograms. A possible explanation is that such time periods are necessary for binding of radioactive 5-HT to subcellular components. The results of the chemical assay experiments indicated that appreciable quantities of 5-HT (approximately 0.1 µg in an intact snail brain) are present in the nervous tissue after exposure to this substance for relatively short time periods (i.e. ½ hr). Much of the 5-HT is presumably contained in extracellular spaces. It is also possible that 5-HT is present 'free' in the cytoplasm for some time before being bound. (In relation to this MAO does not appear to be active on 5-HT in Helix pomatia nervous tissue; see introduction). It is also likely that 5-HT-containing neurons in vivo contain a near maximal complement of 5-HT, and that some time will have to elapse before sufficient nerve activity has taken place for adequate turnover of 5-HT, and hence allow adequate uptake of the exogenous 5-HT supplied in the present experiments.

Finally it is possible that some of the radioactivity localized in nerve endings is due to a metabolite of 5-HT. There is at present no proof that this is not the case. Once again, however, this would seem unlikely because nervous tissue of H. pomatia appears to lack enzymes capable of inactivating 5-HT.

It is thought that a re-uptake mechanism may be at least partly responsible for inactivating 5-HT in mammalian brain (see Iversen, 1971). Mammalian brain slices and synaptosome preparations are capable of an active uptake of 5-HT (Ross and Renyi, 1967; Blackburn, French and Morrills, 1967), and it has been suggested that this occurs in 5-HT-containing nerve terminals (Iversen, 1970; Aghajanian and Bloom, 1967; Bloom and Costa, 1971). In no situation, however,

has it been proved that a nerve ending(s) labelled by exogenously administered 5-HT does in fact normally contain 5-HT.

In contrast to the present work, Coggeshall (1972) has shown that the 5-HT-containing perikarya of the Retzius cells of the leech (see Rude, Coggeshall and Van Orden, 1969), are selectively labelled after exposure to radioactive 5-HT. The reason for the difference is not yet clear. However this finding would appear somewhat unique, for 5-HT-containing cell bodies in the mammalian brain are not labelled by intraventricular injections of radioactive 5-HT (Bloom and Costa, 1971), and neither are neuronal perikarya of Aplysia after exposure to this substance (Ascher et al., 1966).

In summary, the results presented above suggest that there is an active uptake and binding of 5-HT by a small proportion of nerve endings in the nervous system of Helix pomatia. Some of these endings are present in areas of the animal thought to contain serotonergic synapses (i.e. endings of the GSC). Thus it would appear that re-uptake is a likely mechanism for the inactivation of 5-HT in the central ganglia of Helix, as is thought to occur in the heart of Aplysia (Taxi and Gautron, 1969; Carpenter et al., 1971). The situation would seem similar, furthermore, to that which is thought to occur at the noradrenergic synapse (Iversen, 1971).

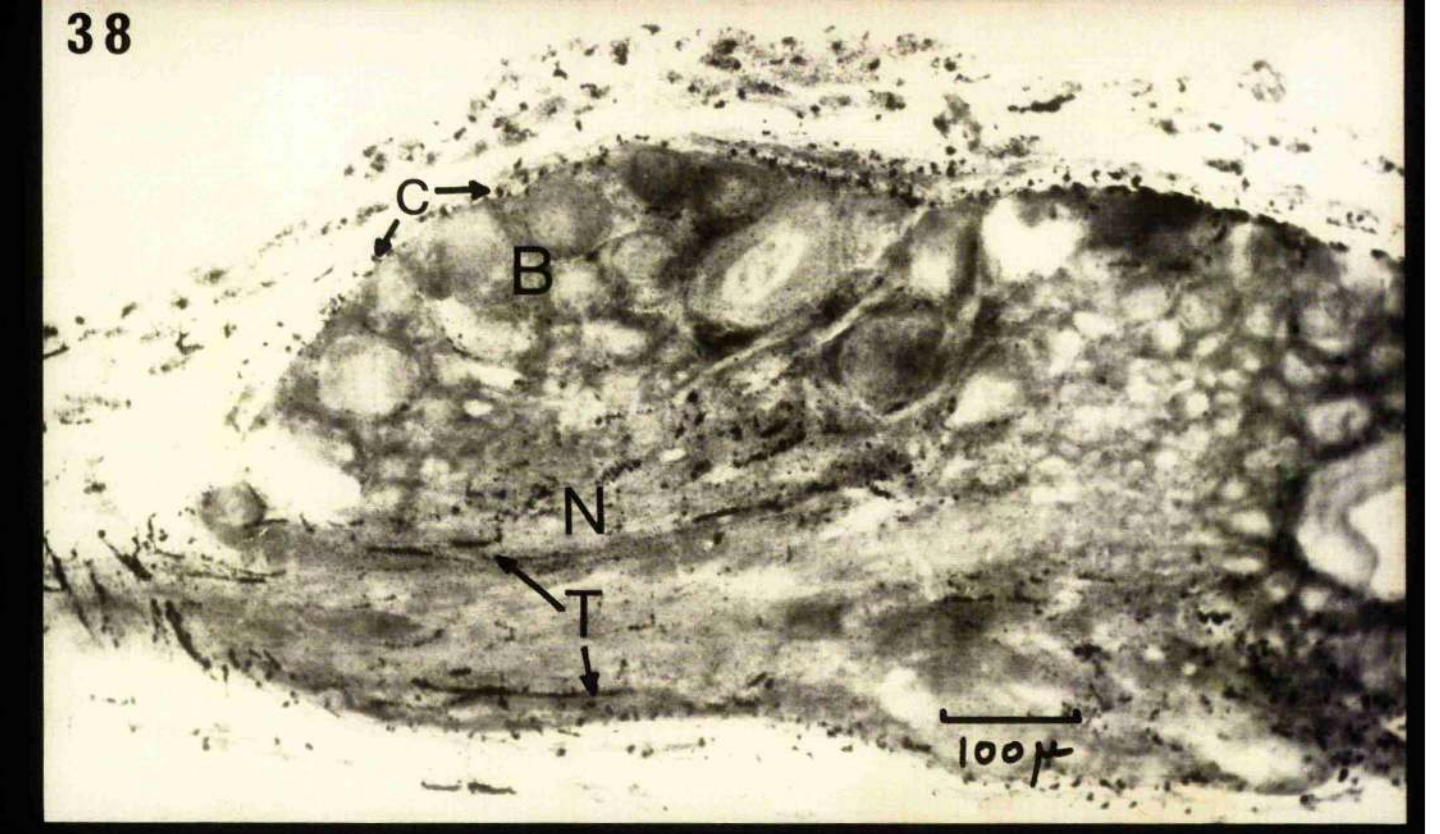
However, further experiments will need to be done to establish whether the endings in H. pomatia which take up 5-HT do in fact normally contain this amine. This point could be tested by injecting labelled compounds into the GSC perikarya and allowing such substances to pass to the endings before completing autoradiographic experiments.

Fig. 38. Autoradiograph of a section of a buccal ganglion of H. pomatia showing the distribution of structures labelled with 5-^{3}HT. Cell bodies (B) are not labelled, whereas many axons and presumed nerve endings in the neuropile (N) are labelled. A nerve tract (T) runs through the bottom of the ganglion. Several labelled structures in this tract are axons sectioned longitudinally (e.g. T, arrows). Many structures in the connective tissue (C, arrows) are also labelled. It is not known if all such structures are nervous, but it is likely that they are because in those areas of connective tissue examined by electron microscope autoradiography, radioactivity was confined to axons.

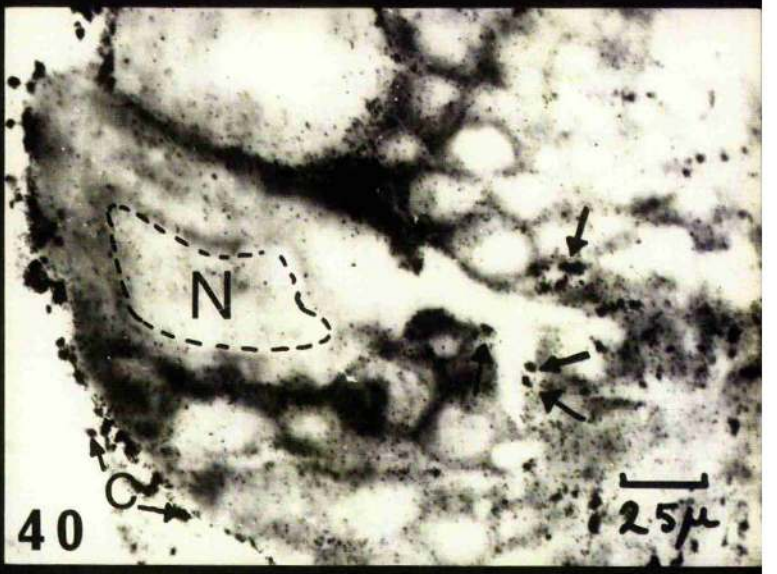
Fig. 39. Light microscope autoradiogram showing an oblique section through the edge of a buccal ganglion. The preparation had been exposed to 5-^{3}HT. Part of a nerve leaving the ganglion is on the right of the micrograph (N). Connective tissue (C) lies to the left of this. Many of the labelled structures in the connective tissue appear to be varicose axons (arrows).

Fig. 40. Light microscope autoradiogram showing a part of a buccal ganglion previously exposed in vivo to 5-^{3}HT. The giant neuron (nucleus outlined) is thought to be synapsed onto by the GSCs. Sites of intense radioactivity are present at the edges of the hillock and main axon branches of this giant buccal neuron (arrows). Other sites of radioactivity are present within the neuropile. Cell bodies are not labelled. The labelled sites in the thin layer of connective tissue around the edge of the ganglion (C, arrows) are probably axons cut in cross section (see Figs. 48, 49).

Fig. 41. Light microscope autoradiogram showing the sparse distribution of nerves which are labelled with 5-^{3}HT in the lip musculature of Helix pomatia. The distribution of silver grains over some nerves (arrows) gives a varicose appearance.


38
C
B
N
T
100μ


39
C
C
C
100μ


N
C
40
25μ


100μ
41

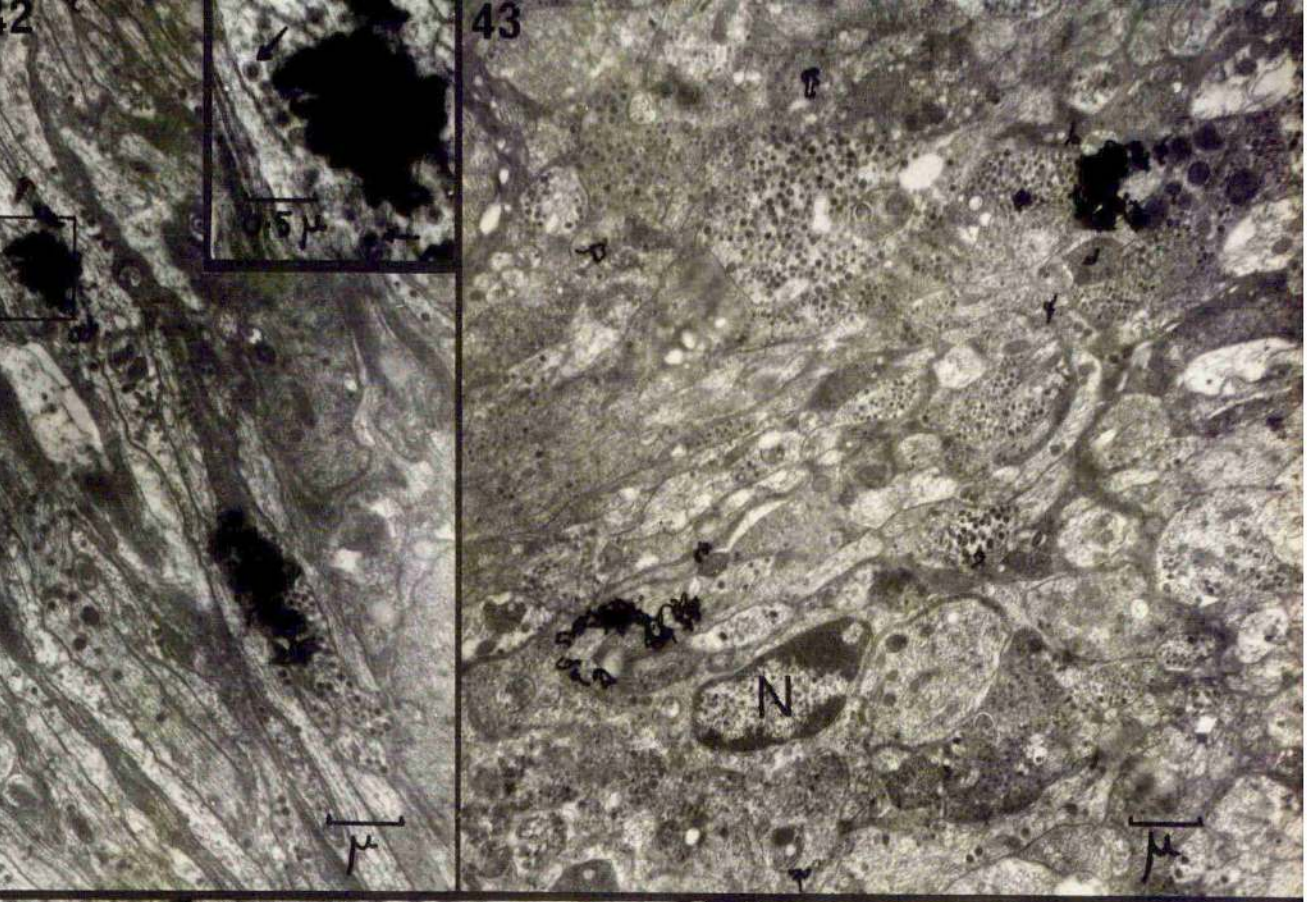
Fig. 42. Electron microscope autoradiograph of part of the neuropile in a buccal ganglion of H. pomatia. Two obliquely sectioned axons are heavily labelled with 5-^{3}HT. Such axons contain granular vesicles (e.g. arrows in the magnified inset).

Fig. 43. Electron microscope autoradiograph of a part of a buccal ganglion neuropile of Helix pomatia previously exposed to 5-^{3}HT in vivo. The micrograph shows, at relatively low magnification, a representative area of the neuropile and illustrates the remarkable selectivity of the uptake process. Only two axonal processes in the area shown are significantly labelled. Both these processes contain dense-cored vesicles. N, nucleus of presumed glial cell.

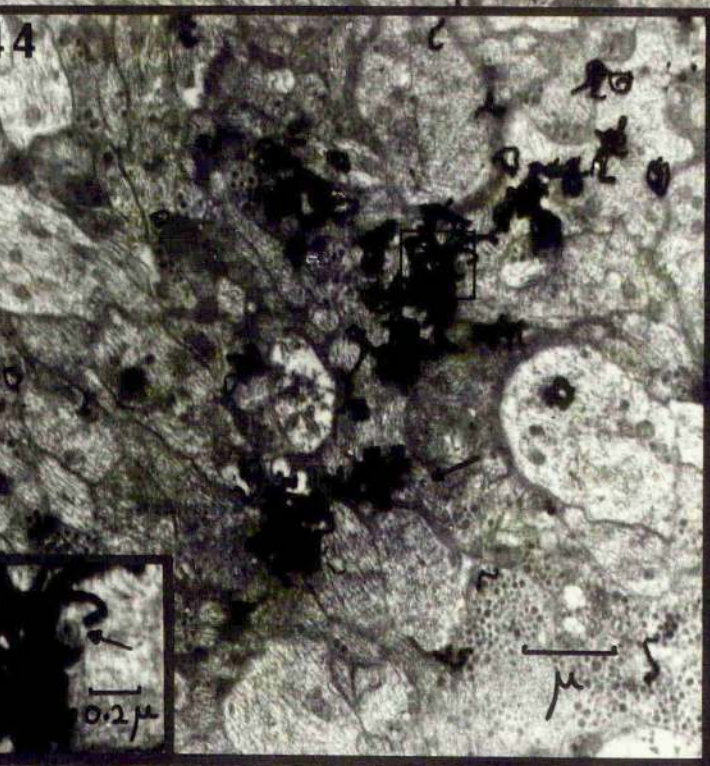
Fig. 44. A branching axon or nerve ending in the neuropile of a buccal ganglion previously exposed to 5-^{3}HT. Although the precise loci of radioactivity is masked by silver grains, this nerve ending contains dense-cored vesicles which are very similar to those in the perikarya of the GSC (e.g. arrows, and inset).

Fig. 45. Electron microscope autoradiograph of a section of the neuropile in a buccal ganglion of Helix pomatia which has been exposed to 5-^{3}HT. Silver grains are localized over one nerve ending which has been selectively labelled. This nerve ending contains dense-cored vesicles of mean diameter 100 nm (arrows) which appear similar to those found in the GSC perikarya.

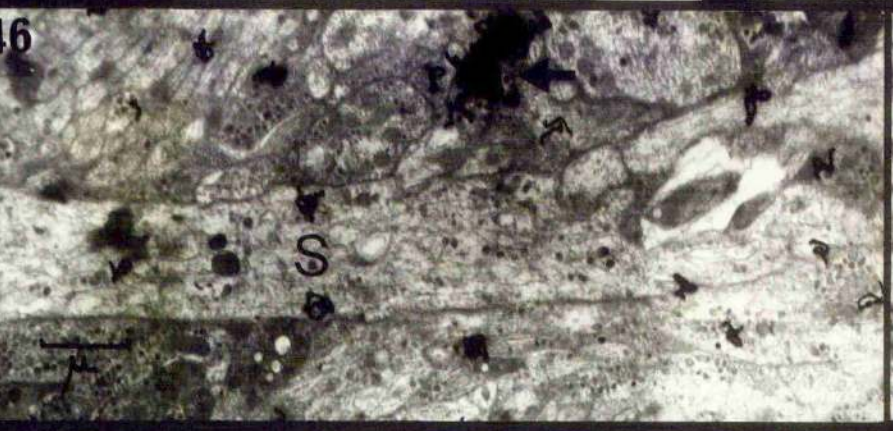
Fig. 46. The axon (S) gives rise to a fine branch (arrow) which is heavily labelled after exposure to 5-^{3}HT. At higher magnification (inset) it is possible to visualize the dense-cored vesicles of mean diameter 100 nm (arrows) which underlie the silver grains.


42
0.5μ
μ

43
N
μ


44
0.2μ
μ


45
μ


46
S
μ

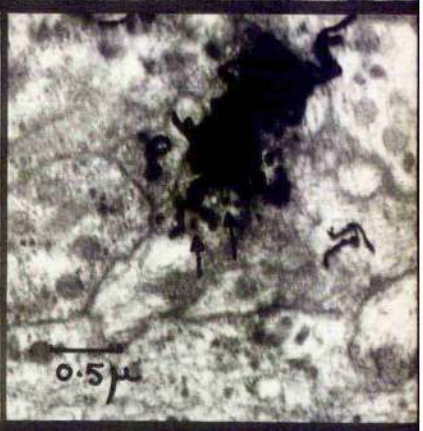
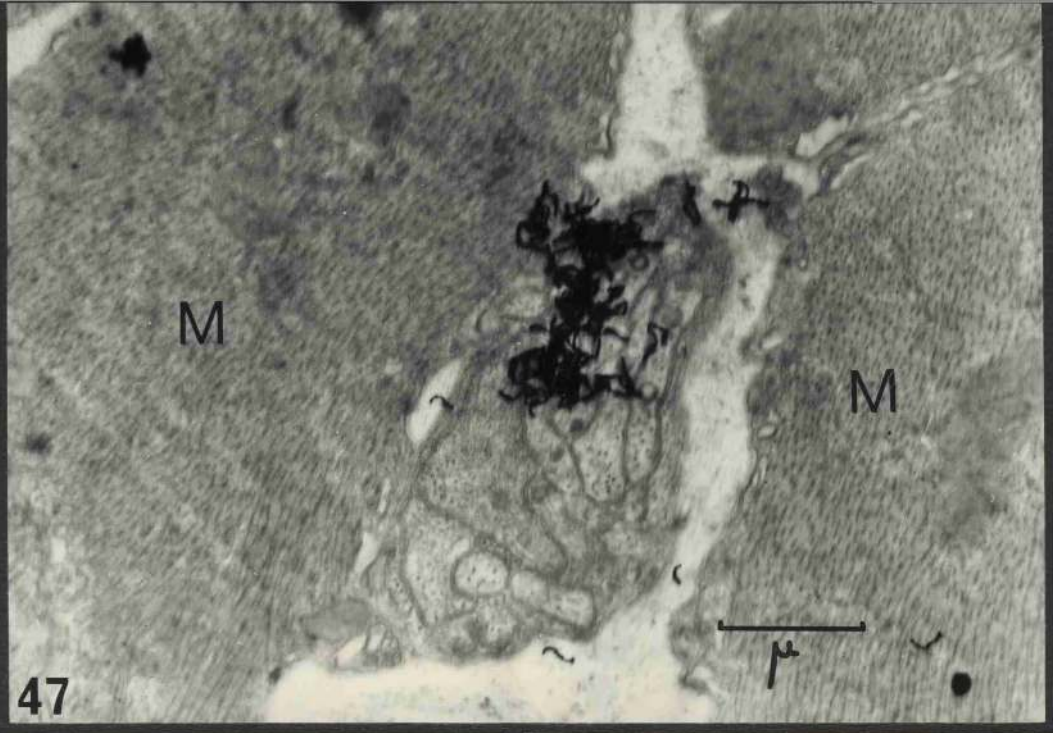

0.5μ

Fig. 47. Electron microscope autoradiograph of a small group of axons running between two muscle cells (M) in a lip of H. pomatia. Only one of the axons in the group appears to be labelled with 5-^{3}HT. This axon runs close to a muscle cell. Because of the masking by silver grains it is not possible to determine the precise arrangements of the membranes or the constituents of the heavily labelled axon.

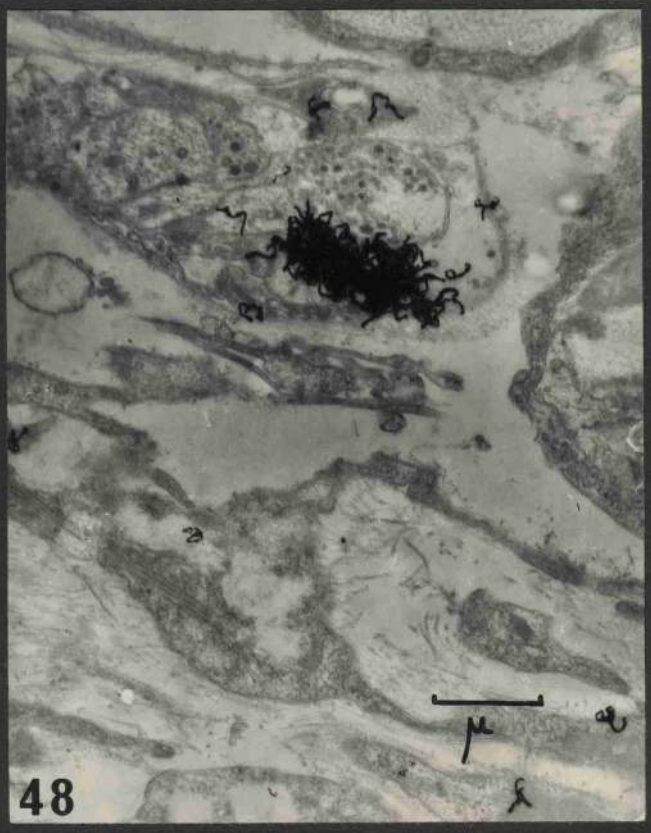
Fig. 48. Electron microscope autoradiograph of a group of axons in the connective tissue sheath of the external lip nerve of Helix pomatia. One of the axons in the group is intensely labelled with 5-^{3}HT. It is not possible to make out which organelles are labelled because of masking by the silver grains.

Fig. 49. A heavily labelled axon in the connective tissue sheath of a cerebral ganglion exposed to 5-^{3}HT. Some dense-cored vesicles of mean diameter 100 nm can be seen (arrows).

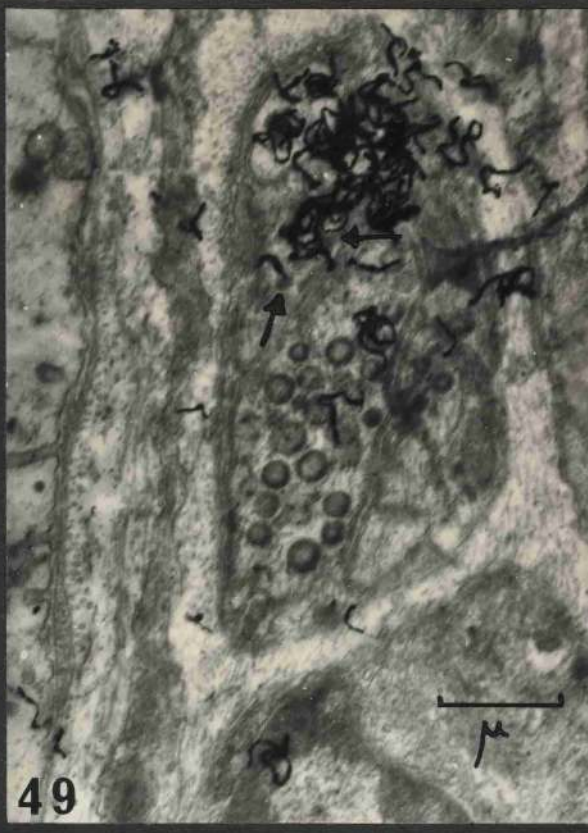
Fig. 50. Light microscope autoradiograph showing uptake of 5-^{3}HT by axons in the external lip nerve of H. pomatia (nerve shown in cross-section). Only a small number of axons are significantly labelled (cf. Fig. 66). Many structures in the connective tissue sheath are also labelled.



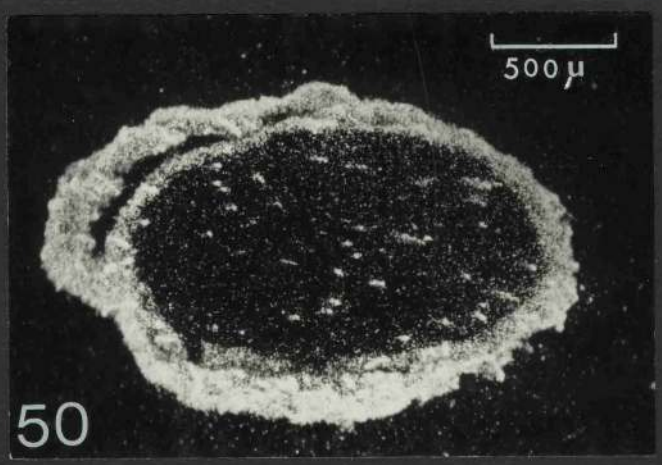
47



48



49



50

Chapter 4

THE UPTAKE OF 5-HYDROXYTRYPTOPHAN AND TRYPTOPHAN BY 5-HT-CONTAINING AND OTHER NEURONS IN *Helix pomatia*

Introduction

If 5-HT serves a transmitter function, it would appear important to understand the mechanisms which control the synthesis and transport of this substance to the nerve endings where it has its transmitter action. Although there is little data on these mechanisms in molluscs (see introduction), and other invertebrates, much information is available on the synthesis of 5-HT in the mammalian nervous system (reviews by Wurtman and Fernstrom, 1972; Glowinski, 1972). For clarity, a brief summary of certain aspects is given here.

The reasons that tryptophan, not 5-HTP, is thought to be the circulating amino acid precursor of 5-HT in the mammalian brain are as follows: (1) under normal circumstances, measurable quantities of 5-HTP cannot be detected in the blood; (2) there is little similarity between the regional distribution of endogenous brain 5-HT, and the pattern of the increment in 5-HT content that follows administration of 5-HTP (Moir and Eccleston, 1968); (3) brains of eviscerated rats can synthesize 5-HT from administered L-tryptophan (Weber and Horita, 1965); (4) the enzyme tryptophan hydroxylase, which converts tryptophan to 5-HTP, has been identified in mammalian brain (Gal, Poczick and Marshall, 1963; Gal, Armstrong and Ginsberg, 1966; Grahame-Smith, 1964a, b, 1967, 1971; Lovenberg, Jéquier and Sjoerdsma, 1967; see also Wurtman and Fernstrom, 1972, and Peters, McGeer and McGeer, 1968 for other refs.). The enzyme is active *in vivo* (e.g. Grahame-Smith, 1971; Jéquier, Lovenberg and Sjoerdsma, 1967; Airaksinen, Giacalone and Valzelli, 1968), and in synaptosome fractions of mammalian brain (Grahame-Smith, 1967; Karobath, 1972); (5) the distribution of tryptophan hydroxylase parallels that of 5-HT in the brain

(Peters, McGeer and McGeer, 1968).

5-HTP formed from tryptophan is decarboxylated by 5-HTP decarboxylase (or more accurately aromatic amino acid decarboxylase, since this enzyme has recently been shown to have the same identity as DOPA decarboxylase; see Christenson, Dairman and Udenfriend, 1972; Wurtman and Fernstrom, 1972) to 5-HT (Fig. 1).

It is thought that both *in vitro* and *in vivo* tryptophan hydroxylation is the rate-limiting step in the biosynthesis of brain 5-HT (Green and Sawyer, 1966; Moir and Eccleston, 1968), whereas the decarboxylation of 5-HTP is never saturated, and has no limiting effect. However the mechanism(s) by which tryptophan hydroxylation is rate-limiting is not clearly understood. On the one hand it has been suggested that a mechanism of negative feed-back inhibition may be responsible (see Macon, Sokoloff and Glowinski, 1971). Alternatively it is possible that, because tryptophan hydroxylase is normally unsaturated with substrate (Eccleston, Ashcroft and Crawford, 1965), plasma tryptophan levels may be a controlling factor (Knott and Curzon, 1972). In relation to this, Grahame-Smith and Parfitt (1970) have proposed that tryptophan transport across the nerve cell membrane may be a controlling factor in 5-HT synthesis. It has also been suggested that because tryptophan hydroxylase requires oxygen (and other co-factors such as a reduced pteridine and ferrous iron; see Lovenberg, Jéquier and Sjoerdsma, 1968), changes in tissue oxygen levels may control the enzyme directly (Diaz, Ngai and Costa, 1968). However, Bloom and Costa (1971) consider that changes in tissue oxygen levels which accompany changes in 5-HT reflect metabolic changes which are independent of tryptophan hydroxylase.

More data will be necessary before the control of brain tryptophan hydroxylase is fully understood. However it is likely that many biochemical factors, including tissue oxygen, accessibility to tryptophan substrate, and feedback influence may control 5-HT biosynthesis (Bloom and Costa, 1971). Furthermore there are other possible controlling factors such as nerve activity (e.g. Aghajanian, 1972, has recently shown that peripheral tryptophan admini-

stration decreases the firing of central 5-HT neurons), supply of substrate by glial cells (see Henn and Hamberger, 1971), external concentration of ions (e.g. Margolis and Lajtha, 1968, have shown that neuronal uptake of glutamic acid, the precursor to γ-aminobutyric acid (GABA), is dependent on the concentration of sodium ions), or alternative enzyme pathways (e.g. Tsuda, Noguchi and Kido, 1972, consider that 5-hydroxytryptophan pyrrolase, an enzyme which cleaves the pyrrole ring of 5-HTP, may be important in the control of 5-HT synthesis).

Although the data provide valuable evidence that tryptophan hydroxylase is a major step controlling 5-HT synthesis, they do not provide information on the intraneuronal loci of the enzyme (i.e. is it present in perikarya, axons, nerve endings, or glial cells, or several of such loci). Because synaptosome fractions of mammalian brain have such enzyme activity (Grahame-Smith, 1967; Ichiyama, Nakamura, Nishizuka and Hayaishi, 1970; Karobath, 1972), it does not mean that nerve endings are the loci of the enzyme. The properties of the tissue may have changed during the isolation procedure. Furthermore synaptosome fractions are a heterogeneous mixture of, for example, cholinergic, noradrenergic and 5-HT-containing nerve endings (see p 43 of Cooper, Bloom and Roth, 1970).

Another subject on which there is little information is the transport of 5-HT along axons following its possible synthesis in neuron perikarya in the mammalian brain. Dahlström and Fuxe (1965) have, however, obtained histochemical evidence which indicates that axoplasmic transport of 5-HT might exist. Thus it is possible that a transport mechanism similar to that of noradrenaline in sympathetic nerves (see Dahlström, 1971) may take place.

The present chapter employs autoradiographic techniques to study the uptake of tryptophan and 5-HTP with respect to identifiable 5-HT-containing neurons (e.g. the CSC) and other non-5-HT-containing neurons, in the CNS of Helix pomatia. The scant data on the role of these substances is precursors to 5-HT in molluscs has been described in the introduction (p 3). Although there is no reason to believe that information obtained from studies of the mammalian brain will prove

to be comparable with that yet to be obtained in molluscs, the data summarized in the previous paragraphs illustrate some of the difficulties that may be expected.

This study attempts to obtain information on the following points: (1) Is there a selective uptake of tryptophan, or 5-HTP by 5-HT-containing neurons in Helix pomatia in vivo? (2) If taken up, do such substances enter neurons directly, or via, for example, glial cells? (3) Is there a transport of such substances, or their anabolic (i.e. 5-HT) or catabolic products, along the length of the neuron once taken up? Previous studies have shown that the CSC in Helix pomatia can synthesize 5-HT from 5-HTP both in vitro (Cottrell and Powell, 1971), and in vivo (Osborne, 1972a), whereas non-5-HT-containing neurons do not.

Materials and Methods

Only active specimens of Helix pomatia were employed experimentally.

Uniformly tritium labelled L-tryptophan (2.0 Ci/m mol) and DL-5-HTP (2.3 Ci/m mol) were obtained from the Radiochemical Centre, Amersham, England. Radioactive contaminants as measured by paper chromatography were less than 2% of the total radioactivity for each substance.

The methods employed were the same as those described in the previous chapter; i.e. for the in vivo experiments brains were perfused via the anterior aorta with saline containing 0.5-1.5 nM labelled tryptophan or 5-HTP. For the in vitro experiments isolated ganglia with their connective tissue sheaths removed were pinned out in small dishes of saline containing 1.0-1.5 nM radioactive tryptophan or 5-HTP. Tissues were then fixed in glutaraldehyde and osmium mixtures and processed for light and electron microscope autoradiography (see chapter 3).

The type and number of autoradiographic experiments completed were as follows: (1) With 5-^{3}HTP, 8 light microscope experiments with isolated central ganglia. 2 electron microscope experiments were made with individual cerebral ganglia whose contralateral cerebral ganglia were found previously to have

produced good autoradiograms at the light microscope level. (2) With ^{3}H--tryptophan, 2 light microscope experiments with intact (perfused) preparations. Electron microscope experiments were not made with this substance for reasons explained in the results.

In addition the amounts of radioactive 5-HTP retained in individual neurons (i.e. the GSC), and in the various ganglia, after exposure to this substance was estimated by liquid scintillation. The methods used were the same as those described in chapter 3.

Results

1. Selective uptake of 5-HTP by identified 5-HT-containing neurons

Only a small number of neurons were labelled after exposure to 5-^{3}HTP either in vivo or in vitro. Comparison with fluorescence histochemical data suggests that all such cells normally contain 5-HT.

The GSC was heavily labelled after 10 hr perfusion of the central nervous system with 5-^{3}HTP. With the light microscope, label was found to be uniformly distributed in the cytoplasm of the cell body, but not in the nucleus (Fig. 51), and to extend along the axon of the cell (Fig. 52). It was possible to trace the labelling along some of the main axon branches through the cerebral ganglia in serial sections (Fig. 54).

With the electron microscope it was not found possible to associate radioactivity in the GSC with any particular organelle. Occasional silver grains appeared to be associated with dense-cored vesicles (mean diameter 100 mμ), which are thought to sequester 5-HT. However, the majority of such vesicles had no silver grains associated with them (Fig. 56). Silver grains were sometimes seen over Golgi structures (Fig. 56). However, the majority of silver grains were not obviously associated with any particular organelle.

Other identifiable neurons thought to contain 5-HT on grounds of fluorescent studies also took up 5-^{3}HTP. A group of such neurons is distributed around the edges of the right, but not the left, cerebro-pedal commissure (Sedden, Korkut and Walker, 1968). The cytoplasms of these cells were heavily

labelled (Fig. 53). Certain large cells in the sub-oesophageal complex of ganglia also selectively took up 5-^{3}HTP. Although these cells were not identified with respect to their position, their number and distribution were similar to those observed by Dahl et al. (1966) to show specific 5-HT fluorescence.

No evidence was obtained that any cell bodies other than those normally containing 5-HT were labelled. Neurons which are known from histochemical fluorescence and bioassay experiments not to contain 5-HT (i.e. the giant cells in the buccal ganglia which are synapsed onto by the GSC, and the cells of the mesocerebrum; see Cottrell and Powell, 1971; Osborne, 1972a) gave no autoradiographic grain counts above background.

Certain structures in the connective tissue sheath surrounding each ganglia and nerve (Fig. 53), and within the neuropile of each ganglion were also labelled. Electron microscope studies were not undertaken to determine the nature of these radioactive structures, but it is possible that those in the neuropile are axons of labelled 5-HT-containing neurons. No label appeared to be associated with glial or muscle cells.

The results of experiments in which the amounts of radioactive 5-HTP retained in single neurons and in whole ganglia were measured, again generally supported the autoradioagraphic experiments. After snail ganglia had been exposed to 5-^{3}HTP *in vitro* for 12 hr, each GSC contained an average of 1 nCi of radioactivity, whereas each non-5-HT-containing giant buccal neuron (Osborne, 1972a) contained a near background count of one tenth this value (4 experiments, 6 cells of each type per experiment). This amount of radioactivity is equivalent to 60 pg 5-HTP per GSC; although some or all of the radioactivity may be due to 5-HT synthesized from 5-HTP (see Cottrell and Powell, 1971), or another metabolite of 5-HTP. The entire brain of *Helix pomatia* contained an average of 0.1 μg of labelled 5-HTP after 12 hr exposure to this substance.

2. General uptake of tryptophan by neurons

After 15 hr exposure to ^{3}H-tryptophan *in vivo*, all nerve cell perikarya in the central ganglia of Helix pomatia were heavily labelled (Fig. 55). No

significant difference in the density of silver grains over 5-HT-containing or non-5-HT-containing neurons was observed. Because of this electron microscopic studies were not undertaken.

Under the experimental conditions used, radioactivity appeared to be chiefly confined to nerve cell parikarya. Silver grains were not seen over axons, as was the case with 5-^{3}HTP over the CSC axons. Very few labelled structures were observed in neuropile (Fig. 55) or in connective tissue; it is not possible to ascertain if such structures are nervous without further electron microscopical evidence.

Discussion

At the present time little is known of the mechanisms by which the precursors of 5-HT, and 5-HT itself, are transported into, and within, neurons. This is especially the case in molluscan nervous tissue.

Although it has been generally assumed that 5-HTP is the blood precursor to 5-HT in molluscs (e.g. Welsh and Moorhead, 1959; Kerkut, Sedden and Walker, 1967), it has recently been claimed that central ganglia of Helix pomatia can hydroxylate tryptophan in vitro (Gardot, 1972). However, Osborne (1972c) has shown that small amounts of both 5-HTP and tryptophan are present in the blood of Helix aspersa, thus it is possible that 5-HTP may be a circulating precursor to 5-HT in peripheral nerves (Osborne and Cottrell, 1970). The latter workers showed that ligatured visceral nerves of Helix pomatia accumulated 5-HT-specific fluorescence on the side of the ligature nearest the visceral ganglion, which suggests a proximo-distal flow of 5-HT along axons with respect to this ganglion.

The results of the present work suggest that the uptake of 5-HTP is selective for 5-HT-containing neurons, whereas tryptophan is taken up by all neurons in the CNS of Helix pomatia.

However, the autoradiographic procedures employed to study the uptake of these substances have limitations which are similar to those described for the uptake of 5-^{3}HT in the previous chapter: One problem is whether or not the labelling of 5-HT-containing cells after 5-HTP administration is due to

5-HTP, 5-HTP undergoing conversion to 5-HT, or an insoluble metabolite of 5-HT or 5-HTP. Two pieces of evidence indicate that the labelling is due largely to 5-HT. First, the GSCs of Helix pomatia can convert 5-HTP to 5-HT in vivo (Osborne, 1972a). This conversion reaches a maximum rate after 2 hr; while perfusion periods of over 2 hr were employed in the present experiments. Second, the histochemical procedures wash out essentially all labelled 5-HTP, but retain bound 5-HT (Gerschon and Ross, 1966).

Another point on which there is no precise data is whether the radioactivity seen in the main axon branches of the GSCs after exposure to 5-HTP results from local uptake, or from transport from the cell body. However, because Osborne and Cottrell (1970) have shown that there is a centrifugal flow of 5-HT along the visceral nerve of Helix pomatia, a flow along the axons of the GSCs from the perikarya would seem probable. This does not of course rule out the possibility that some 5-HTP can also be taken up by parts of the axon. However, no labelling was observed in nerve endings.

It was not possible at the ultrastructural level to determine an association of radioactivity with one particular organelle after exposure to 5-HTP. In the Retzius cells of the leech, vesicles with electron dense cores that are thought to contain 5-HT (Rude, Coggeshall and Van Orden, 1969) are distributed in localized areas of the cytoplasm. These areas are more heavily labelled than the rest of the Retzius cells' cytoplasm following incubation with labelled 5-HTP and 5-HT (Coggeshall, 1972). Thus in this situation there is some direct evidence that 5-HT and/or 5-HTP is associated with the granulated vesicles. In the GSCs of H. pomatia, on the other hand, the granulated vesicles appeared to be randomly distributed in all areas of the cytoplasm, and although all areas of the cytoplasm appeared labelled with the light microscope, there was no definite correlation between silver grains and vesicles. However, a proportion of silver grains appeared to be associated with Golgi structures. It is possible therefore, that these structures have some role in the uptake and/or binding of 5-HTP or 5-HT. Previous workers

have suggested that the Golgi apparatus is involved in formation of different types of vesicles (e.g. Bern, Nishioka and Hagadorn, 1961; Coggeshall, 1967).

Another important question is why relatively long time periods (up to 15 hr) of exposure to radioactive 5-HTP are necessary to give good autoradiographic results. The reason(s) for this are not yet clear, but it is possible that such time periods are due to factors similar to those discussed in relation to the uptake of 5-^{3}HT (chapter 3). For example, they may reflect the time necessary for the binding of radioactive substances to subcellular components, or the time necessary for sufficient nerve activity to take place for adequate turnover of 5-HT. The chemical assay experiments indicated that appreciable quantities of 5-HTP, like 5-HT, were present in the central ganglia after exposure to this substance for short time periods. Once again, it is possible that much of this 5-HTP was contained in extracellular spaces.

The highly selective labelling of 5-HT-containing neurons after exposure to 5-^{3}HTP contrasts with their lack of labelling after exposure to 5-^{3}HT. The assay experiments support these autoradiographic findings, because the GSCs contain more radioactivity after exposure to 5-^{3}HTP than after exposure to 5-^{3}HT, whereas the giant neurons of the buccal ganglia and other non-5-HT--containing neurons show little affinity for either 5-HTP or 5-HT. Extra evidence that there are different uptake mechanisms for 5-HTP and 5-HT has been obtained by experiments with imipramine. Cottrell (1971b) has shown that this substance (concentration 3.5×10^{-5} M) blocks by about 80% the uptake of 5-^{3}HT by the perfused brain of H. pomatia, but under the same conditions (i.e. the same concentrations of imipramine and 5-^{3}HTP, and the same perfusion procedure as used in the autoradiographic procedures) blocks by only about 48% the uptake of 5-^{3}HTP (G.A. Cottrell, personal communication). However, because imipramine has less effect on the uptake of 5-HTP than 5-HT by the entire brain, the possibility cannot be ruled out that the lack of labelling in GSC perikarya following exposure to 5-HT is due to insufficient binding of uptaken 5-HT. The data presented in the previous chapter clearly show that

5-HT taken up into nerve endings remains bound during the histochemical procedures, but it is possible that the sites of subcellular binding (presumably the dense-cored vesicles) differ in properties in the perikarya compared with those in the nerve endings, and that the autoradiographic data merely reflect in some measure these differences.

Finally, it is pertinent to discuss the possible roles of tryptophan and 5-HTP as blood precursors to 5-HT in Helix. As mentioned above, Cardot (1971a, 1972) has suggested that central ganglia of Helix pomatia can hydroxylate tryptophan in vitro. On the other hand 5-HTP increases 5-HT-specific fluorescence in several 5-HT-containing neurons in vivo (e.g. Kerkut, Sedden and Walker, 1967), including the GSC (Osborne, 1970; Cottrell and Osborne, 1970). The GSC can convert 5-HTP to 5-HT both in vitro (Cottrell and Powell, 1971), and in vivo (Osborne, 1972a). However, attempts made in association with the present work (also personal communication by P. Gray) to detect the formation of 5-HTP or 5-HT from tryptophan in the GSCs have met with no success. (These experiments were made using the 'Dansyl chloride' micro-method for detecting and identifying amino acids on polyamide layers (see Neuhoff and Weise, 1970). ^{14}C-tryptophan was perfused through the brain of H. pomatia for 8 hr and then, after thoroughly washing with 'cold' saline, the GSCs were isolated and analysed to detect any label in the position of the chromatograms corresponding to 5-HTP or 5-HT). The blood of H. aspersa contains small amounts of both 5-HTP and 5-HT (Osborne, 1972c), but there is no adequate data on the possible presence of these substances in the blood of H. pomatia. Obviously more work employing sensitive biochemical techniques will be necessary to determine which of these substances (if not both) is the natural circulating precursor of 5-HT.

According to Wurtman and Fernstrom (1972), in the mammalian brain "administered 5-HTP should be viewed as a drug; it should be anticipated that circulating 5-HTP would have a different fate from that of the amino acid synthesized within the neurons." The scant data indicate that, (even should this be proven in mammals) this may not be the case in Helix because (1) the

blood of these animals contains detectable 5-HTP, (2) there does not appear to be any barrier between blood and nervous tissue similar to that in the mammalian brain (chapter 2), and (3) identified 5-HT-containing neurons have been shown to convert 5-HTP to 5-HT *in vivo*.

In summary, the results presented above indicate that 5-HTP is selectively taken up from the blood by 5-HT-containing neurons in the CNS of *Helix pomatia*, whereas tryptophan is taken up by all neurons. It seems likely that 5-HTP is converted to 5-HT and subsequently passed down the axons of the 5-HT--containing neurons. It is also possible that some of the tryptophan taken up by the 5-HT-containing neurons *in vivo* is converted to 5-HT, but biochemical evidence for this conversion has yet to be obtained. Many points, such as the time course of uptake and binding of 5-HTP and tryptophan, and the nature of the binding organelles, require further elucidation.

Fig. 51-54.

Light microscope autoradiograms of sections of Helix pomatia nervous tissue after exposure to tritium labelled 5-HTP in vivo. The autoradiograms were photographed using dark ground illumination and the silver grains appear as individual bright specks.

Fig. 51. One of the GSCs at the edge of the metacerebral ganglion. The cytoplasm of the cell has taken up 5-^{3}HTP, whereas surrounding tissues have not. The nucleus of the cell, N, is not labelled. The scale is 100 μ.

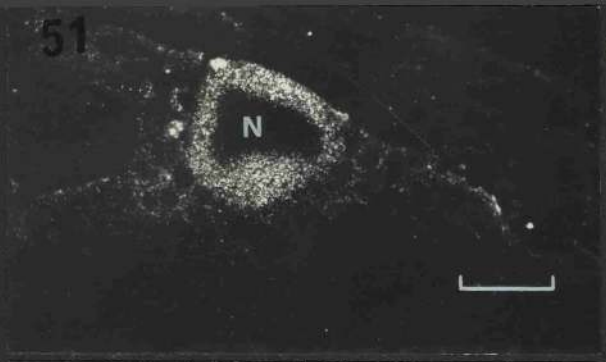
Fig. 52. Autoradiogram showing a longitudinal section of the major axon of a GSC. This axon has been traced from adjacent sections to the soma of the GSC. Some of the activity at the edge of the tissue section, adjacent to the blood sinuses (B), is an artefact caused by stretching of the stripping film over the edge of the section. The scale is 100 μ.

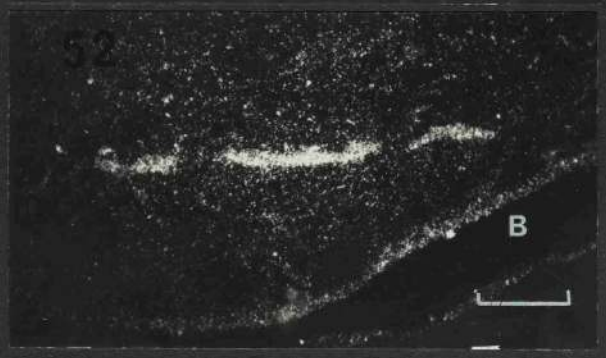
Fig. 53. Autoradiogram of a section through the right cerebro-pedal connective of Helix pomatia. Several neurons around the edge of this connective contain radioactivity in their cytoplasm after in vivo exposure to 5-^{3}HTP. These cells fluoresce specifically for 5-HT. The relatively large nuclei of these cells are not labelled. Certain unidentified structures in the connective tissue layers are also overlain by silver grains. Some of these are likely to be artefacts caused by stretching of the stripping film at the edges of the connective tissue layers (e.g. arrows at the bottom). However no structures are labelled as heavily as the neuron somata. The scale is 100 μ.

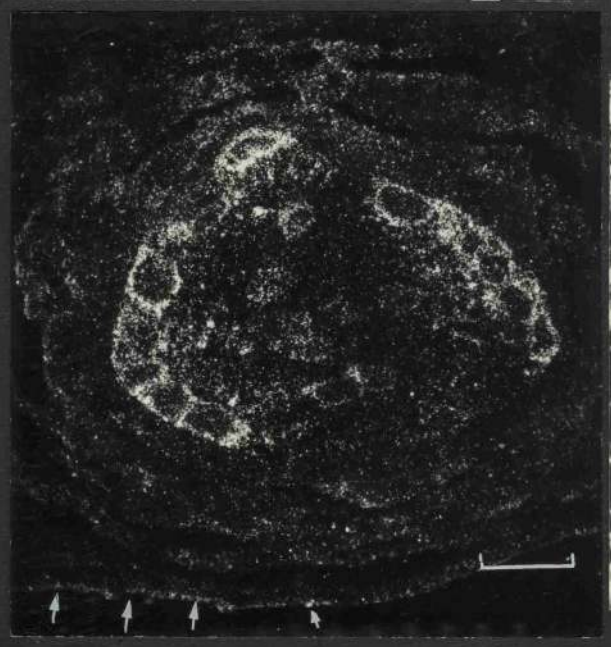
Fig. 54. By serial section autoradiography it is possible to trace the paths taken by the main branches of the GSCs after exposure to 5-^{3}HTP in vivo. Three such 10 μ sections are shown in the micrographs a, b, and c. The arrows point to the sectioned edges of the GSC soma. At the bottom (d) is a reconstruction of the main axon branches of the GSC made from these three sections. The scale in each case is 50 μ.

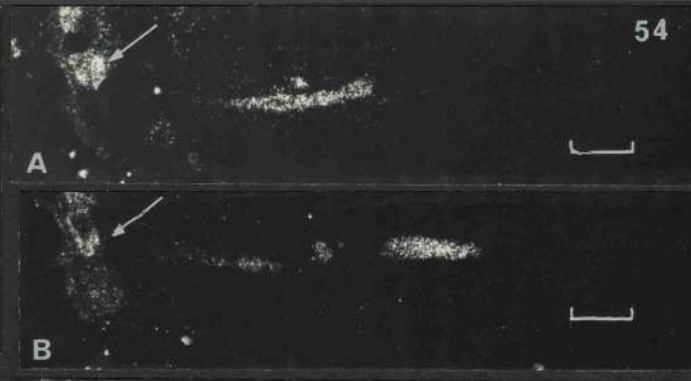
Fig. 55. Light microscope autoradiogram showing distribution of labelled neuron somata after perfusion of the central nervous tissue of Helix pomatia with tritium labelled tryptophan solution. The area shown is a part of the mesocerebrum. These cell bodies do not fluoresce specifically for monoamines, and do not take up 5-^{3}HT nor 5-^{3}HTP. The cytoplasm but not the nucleus (some labelled N) of each neuron somata is labelled. The neuropile of the adjacent metacerebral ganglion (M) has no labelled structures nor has the connective tissue (C). Some cell bodies are more heavily labelled than others. Note the marked absence of labelling over glial cells which surround and lie between all the cell bodies.

Fig. 56. Electron microscope autoradiogram showing a part of a GSC from an animal exposed to 5-^{3}HTP *in vivo*. The micrograph shows an area near the outer edge of a GSC where there are numerous infoldings from glial cells (large arrows). The dense-cored vesicles which are believed to sequester 5-HT (e.g. small arrows) are distributed generally throughout cytoplasm. Some silver grains appear to be associated with Golgi complexes (G), especially at the ends of these structures, where it is possible that the granulated vesicles are formed.


51
N

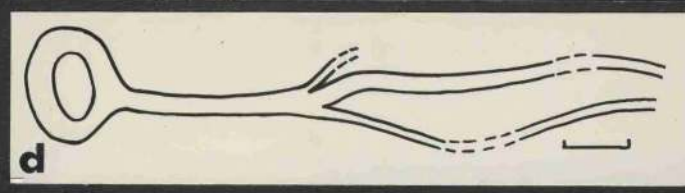

52
B

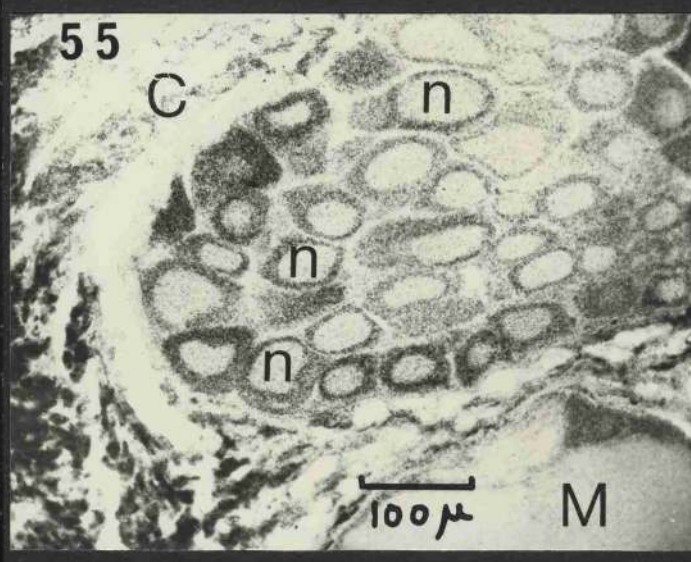


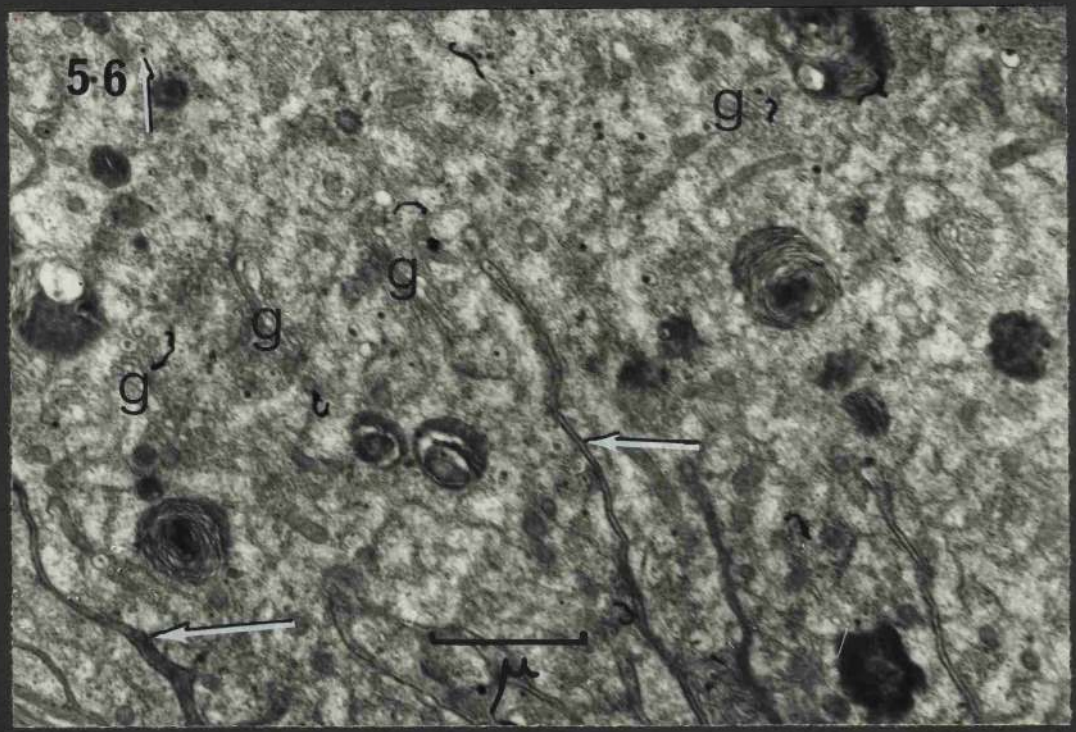

54
A

B


C


d


55
C
n
n
n
100m
M


56
g
g
g
g
.3m

Chapter 5

AN ANALYSIS OF SOME PERIPHERAL AXON BRANCHES OF THE GSC OF *Helix pomatia*, WITH SPECIAL RESPECT TO AXON BRANCHES ENDING ON MUSCLES NEAR THE MOUTH

Introduction

The GSC in each metacerebral ganglion of *Helix pomatia* sends one axon branch into the ipsilateral external lip nerve, another into the ipsilateral cerebro-buccal connective, and a third branch into the contralateral cerebro--buccal connective (Kandel and Tauc, 1966a; see Fig. 2). The branches in the cerebro-buccal connectives form excitatory monosynaptic links with several giant neurons in the buccal ganglion (Gottrell, 1970a, b, 1971a, c).

Because the GSC soma is relatively large, it would seem likely that the neuron has numerous presynaptic endings. Presumably the endings which form synaptic connections with several neurons in the buccal ganglia represent only a very small proportion of the total synaptic field of the GSC. The present chapter describes experiments made to determine the peripheral pathways of the GSC axons in the external lip nerves, and to locate structures innervated by the GSC axons in the region of the lips. Preliminary data is also presented on axon pathways of the GSC which appear to run in nerves leaving the buccal ganglia.

Materials and Methods

(1) Electrophysiology

The cerebral and buccal ganglia, external lip nerves and lips were dissected from active *Helix pomatia*. The preparation was pinned to the plastic base of a small perfusion chamber which contained 3 ml of saline (Meng, 1960). Connective tissue above the GSCs was removed by dissection, and the GSCs were impaled with double-barrel glass microelectrodes containing 2.5 M KCl or 0.6 M Na_2SO_4.

The external lip nerve and its branches, and muscles located at the peripheral ends of the external lip nerve, were held in tightly fitting plastic suction electrodes so that they could be either stimulated or recorded from while still immersed in the saline. Plastic suction electrodes were also used to record from nerves leaving the buccal ganglia.

Signals from the preparation were recorded via conventional cathode followers and differential amplifiers and displayed on a dual beam oscilloscope (Tektronix) or pen recorder (Brush model 220).

In order to block trans-synaptic conduction a ringer containing a relatively high concentration of Mg^{++} but no Ca^{++} was employed. The composition of this ringer was as follows: NaCl, 2.45 g/l, KCl, 0.43 g/l, $NaHCo_3$, 1.10 g/l, $MgCl_2.6H_2O$, 5.50 g/l.

Changes in length of individual or of groups of lip muscles, were recorded by attaching their ends by cotton thread to a balsa wood rod (5-20 cm long), which was glued to the peg of a RCA 5734 mechano-electrical transducer. This sensitive transducer was first employed by Atwood, Hoyle and Smith (1965) for measuring isometric changes in single crab muscle fibres. In the present work small pieces of elastic were attached to the end of the balsa wood rod so that the lip muscles were maintained under tension. In this manner changes were measured approximately isotonically.

(2) Histology

Lip muscles were processed for fluorescence histochemistry by the method of Falck and Owman (1965). The histochemical procedure employed has been described in chapter 1.

The structure of the external lip nerve and lip muscles were studied with the electron microscope. For this pieces of tissue were fixed for 1 hr in 1% OsO_4 in 0.2 M veronal acetate buffer pH 7.4. Fixation was completed at 4°C. Subsequently tissues were dehydrated through a series of acetone-water solutions and embedded in Araldite. Thin sections were stained with lead citrate and uranyl acetate.

(3) Pharmacological tests

Individual lip muscles were dissected from live animals and suspended in a 5 ml organ bath. A thread tied to one end of a muscle was attached to a balanced aluminium lever, and recordings of muscle length were made using a kymograph.

Acetylcholine iodide and 5-HT creatinine sulphate were added to the bathing saline to give final concentrations from 10^{-8} - 10^{-4} g/ml.

(4) Biological assay of 5-HT

The amounts of 5-HT in the external lip nerve, branches of the external lip nerve, and in lip muscles were assayed on the heart of Helix aspersa. This preparation is known to be very sensitive to 5-HT, but relatively insensitive to catecholamines (Osborne, 1970; Cottrell and Osborne, 1969a). The isolated heart was freely suspended via a cannula attached to the auricle, and test solutions were added to the perfusing saline (Meng, 1960).

Extracts of tissue were prepared by homogenization in either Meng's saline or distilled water. Control extracts were identical volumes of saline taken from the dissection bath adjacent to the tissue being assayed.

Results

1. Axon branches of the GSC in the external lip nerve

A small ganglion containing several hundred small neurons (diameters 10-20 μ is situated peripherally on each external lip nerve (Figs. 58, 68). Nerves leading from this ganglion pass to a complicated system of muscles, which control movements of the mouth of the animal, and also to the skin of the lips. Axon branches of the GSC appear to pass through the lip ganglion, and run to the lip muscles in these nerves. The evidence for such axon branches of the GSC is as follows: (i) Stimulation of any small nerve at its point of contact with the muscle triggered an antidromic action potential in the GSC. The antidromic action potential occurred in two steps indicating sequential invasion with an axonal potential (A spike) firing before the cell body (S spike). The axonal potential was made smaller and ultimately blocked by artificially hyperpolarizing

the cell body (Fig. 57). (ii) When the GSC was stimulated directly a small spike could be recorded extracellularly from the nerves close to the lip muscles. The latency between the GSC spike and the recorded action potential was constant for any particular branch of the external lip nerve at about 30 m sec (Fig. 58c), and was the same as the latency of the antidromic potential recorded after nerve stimulation. (iii) There was a one to one relationship between the GSC action potential and peripheral spike after several hundred action potentials, and after GSC firing at high frequency (Fig. 58c). The one to one relationship was not affected by bathing the preparation with snail saline containing high Mg^{++} and no Ca^{++}.

Extracellular recordings from a point halfway along the external lip nerve during direct GSC stimulation showed two interesting features about the axon branch(es) of the GSC. First, the extra-cellularly recorded spike was one of the largest which could be recorded from the external lip nerve (Fig. 58b), when it was compared with spikes resulting from electrical stimulation of the whole nerve. Furthermore there appeared to be no more than five units in the entire external lip nerve which were of a size comparable to that of the GSC. Because of this the external lip nerve was examined with the electron microscope. Under the assumption that spike size would reflect axonal diameter, it was hoped that such examination might show only five or so large axons, one of which might contain dense-cored vesicles similar to those present in the main axon branches of the GSC (chapter 1). If this were the case then it would also be possible to obtain some indirect information about the morphology of a peripheral GSC axon.

Fine structural observations only partially fulfilled these expectations. In cross section the external lip nerve was seen to contain in the order of 10^5 parallel-running axons. The diameters of the majority of these axons were less than 1 μ (Fig. 66). However a very small proportion of axons had diameters greater than 3 μ, and furthermore only approximately six of these had diameters over 5 μ (Fig. 67). Thus there appeared to be a satisfactory correlation

between the electrophysiological and morphological data. On the other hand, it was not possible to identify any features (e.g. dense-cored vesicles of mean diameter 100 nm) within any of the large diameter axons which could be related to those of the main axon branches of the GSC described in chapter 1.

A second feature of the GSC axon potential recorded from the external lip nerve was that it was consistently complex in form. It was not simply biphasic, but exhibited one or more inflections, depending on the precise arrangement of the recording electrodes (Fig. 58a). The shape of the inflection(s) was constant after repeated stimuli for any particular recording position. This suggests that more than one axon branch of the GSC occurs in the external lip nerve, i.e. the branches of the GSC that run in the peripheral branches of the external lip nerve may arise near or within the cerebral ganglion. In relation to this, one of the giant neurons in the buccal ganglion of H. pomatia sends two parallel-running axon branches into the ipsilateral cerebro-buccal connective (G.A. Cottrell, personal communication). Experiments to test this (e.g. using split external lip nerve preparations) were not undertaken. However some experiments were made to find out if axon branches of the GSC were present in the connective tissue sheath of the external lip nerve (this seemed likely because the autoradiographic experiments described in chapter 3 showed that certain axons in this sheath accumulated radioactive 5-HT). These experiments were made by dissecting the sheath from the nerve, and subsequently either stimulating or recording from the sheath while recording from the GSC. The results indicated that no axon branches of the GSC were present in the nerve sheath.

Experiments were made to determine the function(s) of the GSC axon branches which appeared to run to the lip musculature. Electrical activity was recorded extracellularly from the lip muscles innervated by branches of the external lip nerve. In the resting state the electrical activity was composed of muscle potentials which were relatively constant in frequency and amplitude for any particular recording site on any particular muscle. When the GSC was stimulated intracellularly to fire a burst of spikes there was a marked increase in the

frequency of the muscle potentials without any noticeable change in muscle length (Fig. 58d, e). Several GSC spikes were necessary to produce this effect; no increase in electrical activity was observed after a single spike or low frequency firing from the GSC. The increase in muscle potential frequency lasted for several seconds after cessation of GSC stimulation. The effect caused by GSC stimulation was abolished if the external lip nerve was severed, or if stimulating current was passed from a microelectrode removed from the GSC and placed adjacent to the GSC in the bath. The increase in electrical activity caused by GSC stimulation was mimicked by 5-HT (concentration 10^{-6} g/ml) applied locally to the muscles from the tip of a small bore pipette (Fig. 59).

The importance of the increase in electrical activity is not however clear. In no case was any lip movement or change in muscle length observed following GSC stimulation in relatively intact preparations. However some information was obtained by attaching a transducer to the end of individual muscles (see methods). This showed that GSC stimulation slightly increased the rate of muscle relaxation following a contraction induced by direct stimulation of the muscle (Fig. 61). This effect was more marked if the muscle was artificially stretched during its relaxation. Furthermore organ bath experiments with isolated lip muscles indicated that 5-HT (bath conc. 10^{-6} g/ml) slightly relaxed contraction induced by 10^{-6} g ACh/ml bath saline (Fig. 62). Thus it is possible that 5-HT released onto the nerves by the GSC may in some way facilitate muscle relaxation. No effect on lip muscle length was observed if the GSC was made to fire a burst of spikes just before or during direct stimulation of the muscle. Thus the GSC did not appear to enhance or reduce active muscle contraction.

It is also not clear whether the increase in electrical activity is due to 5-HT liberated from endings of the GSC directly onto the lip muscles or onto other neurons. Although axon branches of the GSC were traced to small branches very close to the muscles, it is possible that such axon branches synapse onto other neurons which are in close association with the muscles.

However several pieces of evidence indicate that 5-HT-containing nerve endings are present on these muscles. First, lip muscles processed for fluorescence microscopy have many yellow, apparently 5-HT-specific, fluorescent varicosities along their lengths (Fig. 64). Second, bio-assay experiments using the isolated heart of Helix aspersa showed that the lip muscle tissue contains approximately 0.1 µg 5-HT/g fresh tissue (Fig. 63). It would seem likely that this 5-HT is present in nerves in the muscle tissue. Third, electron microscope autoradiographic experiments have shown that tritiated serotonin is taken up selectively by only a small proportion of axons and nerve endings in nerves which innervate the lip muscles (see chapter 3). Analysis of the lip muscles by intracellular techniques and the use of drugs which modify serotonergic transmission (e.g. imipramine) may resolve whether or not the GSC affects the muscles directly.

2. Axon branches of the GSC in nerves of the buccal ganglia

Preliminary experiments were made, using extracellular recording techniques, to locate possible axon branches of the GSC in nerves of the ipsilateral buccal ganglion. The results are summarized in Fig. 65. Evidence was obtained that only three ipsilateral buccal ganglion nerves contained GSC axon branches. These were as follows: (1) a relatively large axon branch in the posterior branch of the second pharyngeal nerve (Fig. 65c), (2) a small axon branch in the anterior branch of the second pharyngeal nerve (Fig. 65b), and (3) a small axon branch in the posterior oesophageal nerve (Fig. 65a). These results were obtained from orthodromic stimulation of the GSC; experiments using antidromic stimulation of the nerves with hyperpolarization of the GSC (cf. Fig. 57) were not undertaken, and consequently the results do not show definitely that such branches exist. However they strongly suggest that the GSC, besides making synaptic contact with several neurons in each buccal ganglion (Cottrell, 1970a, 1971b), also has extensive axon branches beyond the buccal ganglia. It is interesting that the branches of the second pharyngeal nerve run to muscles in the pharynx, and that there are apparently no aggregates

of neurons on such nerves before they reach the muscles. Thus it is possible that the branches of the GSC which appear to be present in these nerves have a similar effect on the pharyngeal muscles as those in the lip muscles.

Discussion

The data presented above suggest that the GSCs of Helix pomatia give rise to large numbers of axonal branches which are present in the mouth and buccal mass of the animal. There is good evidence that some presynaptic endings of the GSC are present in the buccal ganglia (Cottrell, 1970a, 1971a), and it also appears that some other endings are present in muscles in the lips, and perhaps also in some pharyngeal muscles. Thus the GSC axon branches are distributed amongst parts of the animal involved in feeding.

However the function(s) of the GSC in the feeding of the animal is far from clear. The work of Cottrell (1970a, 1971a) has shown a direct excitatory action on some neurons in the buccal ganglia, but it is not known if such neurons are motor or internuncial. The GSC also appears to excite at least one unidentified neuron (either directly or indirectly), which may be located in the ipsilateral buccal ganglion, and which sends an axon branch in the oesophageal nerve (Fig. 65a). On the other hand, it appears that some axon branches of the GSC alter the rate of firing of lip muscle potentials. Much information will obviously be necessary before the function of these, and the other axon branches of the GSC are fully understood. Consequently it is not at present possible to discuss the role of the GSC as an effector or integrative unit.

The following discussion, however, deals with certain more specific aspects of the GSC (see also Cottrell, 1970a, 1971a, and Gerschenfeld, 1973 for a discussion of the GSC synapses onto neurons in the buccal ganglia).

1. The significance of the increase in lip muscle potentials resulting from GSC stimulation

The evidence presented above suggests that the increase in rate of lip muscle potential firing may be due to 5-HT released onto the muscles by the GSC. Three pieces of work appear important in relation to this suggestion. First,

Hidaka, Osa and Twarog (1967) have shown that exogenously applied 5-HT increases the ability of the membrane of the anterior byssal retractor muscle of Mytilus edulis to fire spikes. The mechanism of this action of 5-HT is thought to be due to an increased Ca^{++} release from the muscle (Bloomquist and Curtis, 1972). Second, a facilitatory effect of 5-HT on neuromuscular transmission has been shown in lobsters (Grundfest, Reuben and Rickles, 1959) and in crayfish (Dudel, 1965). Third, Cooke (1966) has shown that exogenous 5-HT may directly facilitate neuromuscular transmission in the heart of decapod crustacea. Thus it is possible that the GSC may facilitate spike firing in the lip muscles via a similar mechanism to that present in one or more of these situations.

It is not clear, however, how such increase in the lip muscle spike firing can be related to the proposed increase in rate of muscle relaxation. Although the effect of GSC stimulation was mimicked by exogenously applied 5-HT, muscle potential firing was also increased (both in frequency and amplitude) by exogenously applied ACh (concentration 10^{-6} g/ml bathing solution), but in this case with muscle contraction.

Furthermore, the possibility has not been entirely ruled out that the effect of GSC stimulation may be indirect, for example via neurons present in close association with the lip muscles. Obviously more work will be necessary to elucidate the precise role of the GSC axon branches running to the lip muscles. However it should be pointed out that, even should the increase in electrical activity of the lip muscles brought about by GSC stimulation be resolved as indirect, it nevertheless must be considered an ultimate function of this 5-HT-containing neuron.

2. Synaptic input and GSC activity

When the central ganglia of H. pomatia are isolated, and the GSC is impaled with a microelectrode, it normally fires a burst of spikes, but then remains inactive (Cottrell, 1970a). If, however, relatively intact preparations (i.e. leaving the whole buccal mass, mouth, and as much of the foot musculature as possible attached to the ganglia) are employed, the GSC fires occasional spikes.

This relative inactivity of the GSC is puzzling, because artificially applied bursts of activity are necessary to elicit any noticeable change both in the resting potentials of buccal ganglion cells which are innervated by the GSC (Cottrell, 1970a), and in the frequency of lip muscle potentials, or their proposed rate of relaxation. It is possible that even minimal dissection alters the normal excitatory input onto the GSC, and that the activity seen in the experimental preparation is very different from that in vivo. In relation to this the GSC does receive a large field of excitatory input from many nerves entering the cerebral ganglion (e.g. internal, middle and external lip nerves, and cerebral-pleural, cerebral-pedal and cerebral-buccal connectives; see Kandel and Tauc, 1966a), but only a relatively small inhibitory input from two tentacle nerves (Cottrell, Macon and Szczepaniak, 1972; Szczepaniak and Cottrell, 1973). In the present work it was also found that mechanical or electrical stimulation of any point of the ipsilateral or contralateral lip skin invariably elicited excitation in the GSC. Similarly De Vleiger (1968) has shown that mechanical stimulation of the lips of Lymnaea stagnalis results in a marked increase in afferent activity in the lip nerves which run to the cerebral ganglia of this animal. It is possible therefore that much of the excitatory input onto the GSC normally present in intact, active animals is not present in relatively intact experimental animals.

However some evidence was obtained that the GSC may in fact be driven to fire regular bursts of spikes (1 burst every 5-10 min) by some pacemaker within the CNS (see Fig. 60). This phenomenon was observed in several relatively intact preparations which had been employed experimentally for several hours previously. Consequently it is possible that fatigue, damage or general deterioration of the preparation may have produced this artefactually. On the other hand, bursting is now recognized as a normal feature of many gastropod nervous systems, and has been implicated as a trigger for feeding in the buccal ganglia of Planorbis corneus (Berry, 1972). It is also likely that the bursting occasionally seen in the GSC is not due to damage (M.S. Berry,

personal communication); but may, furthermore, reflect a return to the normal some hours after the dissection of the preparation. Obviously more work using relatively intact preparations is necessary; but because it is impossible to record from the GSC of an entirely intact and active animal, the problem of what is the normal activity of the GSC may remain a moot point.

3. A comparison of the functions of the GSC with other identified 5-HT-containing neurons

The only comparable situation in which electrophysiological studies have been made of an identifiable 5-HT-containing neuron are those made on the Retzius' cells of the segmental ganglia of several species of leech. These cells are electrically coupled (Hagiwara and Morita), and have axon branches in the lateral nerves which leave each ganglion (see Lent, 1972). It has been proposed that the Retzius cells are inhibitory motor neurons (Marsden and Kerkut, 1969; Lent, 1971). However it has recently been shown by Lent (1973) that the cells play some part in the control of mucus release from glands which lie in the body wall of the animal. The rate of mucus release increased with the impulse activity of the Retzius cells, and this effect was mimicked when 5-HT was applied locally to the body wall of the animal. It is not however, known whether the process of mucus release is mediated by a direct synaptic effect of endings of the Retzius cells onto mucus glands, or whether the effect is indirect; for example via a neurosecretory route (Lent, 1973). Furthermore there are no data on the innervation of mucus glands (if they are innervated), or the natural mechanism of liberation of mucus from the glands. It is also likely that the control of mucus release is not the only effector role of these large neurons (Lent, 1973). An attempt is being made to find out whether or not the activity of the GSCs of Helix pomatia effects mucus liberation from the lips of this animal.

In the mammalian central nervous system, there is no specific neuron or synaptic connection which has allowed an analysis of the effects of neurally released 5-HT, as compared with the effects of 5-HT administered by micro-pipettes. Nevertheless, there is some data implicating 5-HT-containing neurons

in certain multicellular functions such as temperature regulation and sleep (see General Discussion).

Finally, mention is made here of the perhaps obvious likelihood that the 5-HT-containing neurons of different phyla will have different functions. Furthermore, it would seem likely from the present work that different axonal branches of one particular 5-HT-containing neuron in a particular species may have different functions.

Fig. 57. Intracellular recordings from a GSC of Helix pomatia obtained by stimulating a small peripheral branch of the ipsilateral external lip nerve. At resting potential (RP, top trace) an S spike follows the stimulus artefact. With increasing hyperpolarization of the GSC soma (brought about by passing direct current through one side of the double-barrelled electrode) the S spike disappears (-15 mV), leaving only the axonal spike (A spike). With further hyperpolarization the A spike is reduced (-30 mV), and eventually blocked altogether (-50 mV). The remaining depolarization following stimulation at -50 mV is an epsp. The effect is reversible, i.e. when the cell is returned to its resting potential (RP, bottom trace) the S spike re-appears. There is consistent latency between the stimulus artefact and A or S spike in each trace.

57

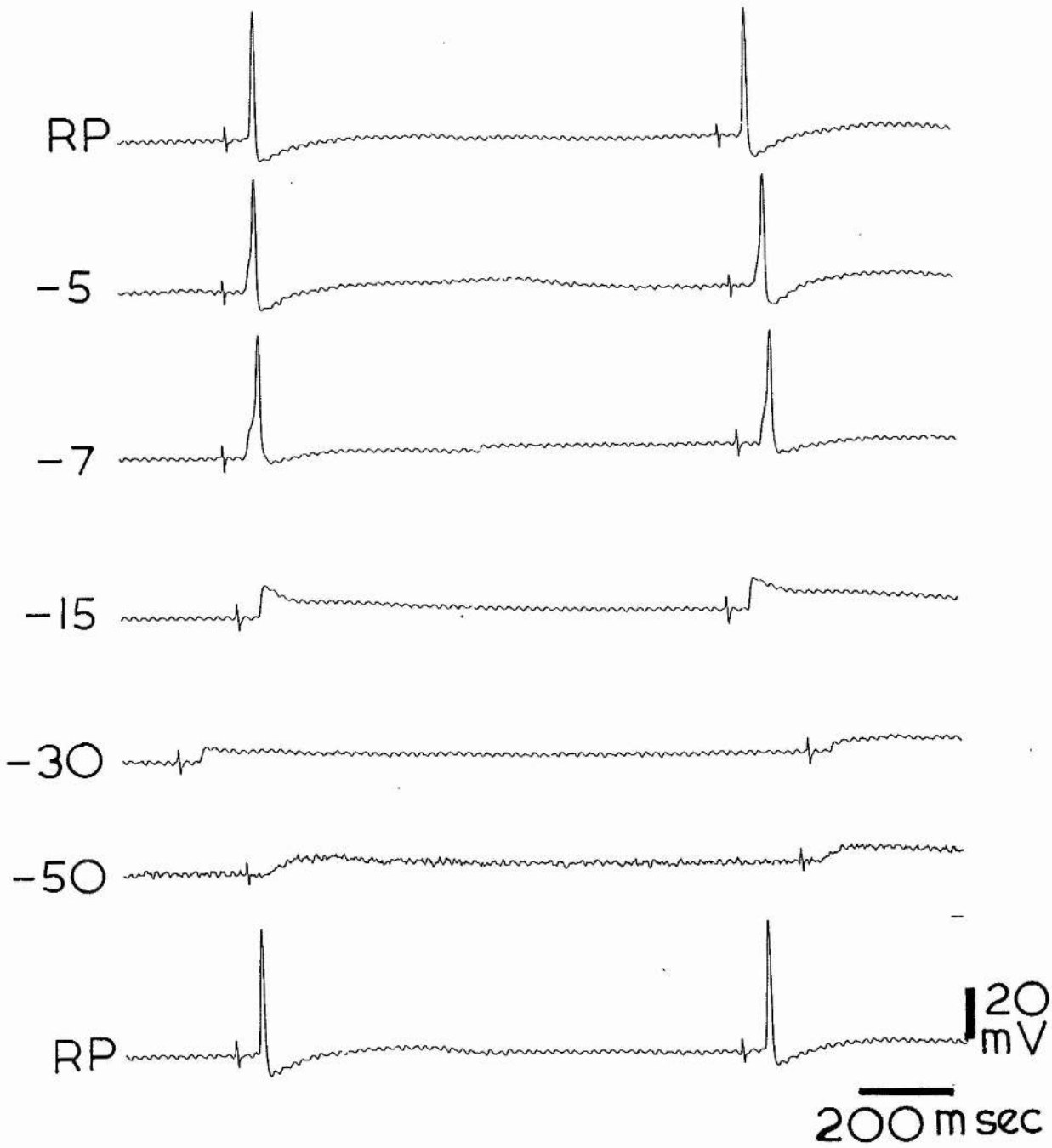


Fig. 58. The diagram (top) shows, approximately to scale, a GSC in the right cerebral ganglion of Helix pomatia, the nerves which contain axon branches of this neuron (eln, external lip nerve. cbc, cerebro-buccal connective. cc, cerebral commissure), and the branches of the external lip nerve which run via a small lip ganglion (lg) to the lip muscles (lm) and lip epithelium (ep). mln, median lip nerve. iln, internal lip nerve. cpc, cerebro-pedal connective.

A and B. Responses recorded extracellulary in the external lip nerve half-way between the cerebral and lip ganglion (top traces), in response to GSC firing caused by a depolarizing pulse (bottom traces). (A) shows that the GSC axon spike is complex in form with several inflections (arrows). The relatively constant shape of this spike indicates that more than one GSC axon branch occurs in the external lip nerve. Time calibration 50 m sec, upper voltage calibration 120 μV, lower voltage calibration 50 mV. (B) shows that the GSC axon spike is large, which suggests that one of the GSC axons is also large. Time calibration 2 sec, upper voltage calibration 100 μV, lower voltage calibration 20 mV. (C) Responses recorded from a small branch of the external lip nerve (top trace) after direct stimulation of the GSC at high frequency. The small spike recorded from this particular branch (arrow) has a constant delay from the GSC action potential of 30 m sec. Time calibration, 50 m sec, upper voltage calibration 20 μV, lower voltage calibration 30 mV. (D and E) The effect of GSC firing (top traces, voltage calibration 20 mV) on the frequency of electrical activity recorded from the lip muscles (middle and bottom traces). The arrangement and relative size of the suction electrodes used to obtain these records is indicated for example by x and y on the top diagram. The difference in shape of the muscle potentials between D (monophasic) and E (biphasic) is due to slight differences in the arrangement of the recording electrodes. Voltage calibration of middle traces is 50 μV. The lower trace in D and E is a plot of the frequency of muscle potentials per second.

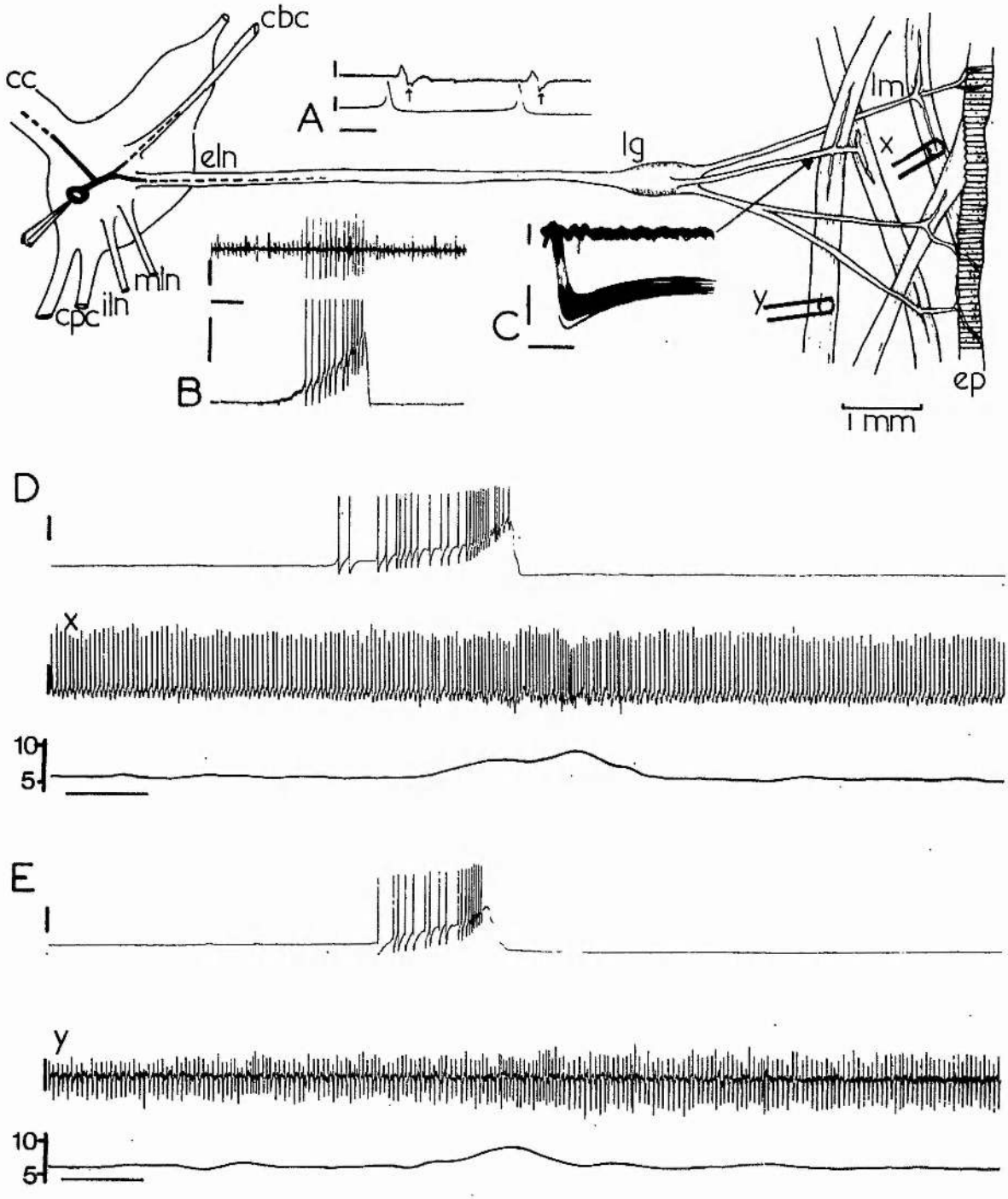

cbc
cc
A
eln
lg
lm
x
mln
cpc
iln
B
C
y
ep
1 mm
D
x
10
5
E
y
10
5

Fig. 59. Record showing the increase in muscle potential frequency caused by 5-HT (10^{-6} g/ml) applied to the muscle from the tip of a small bore pipette. The effect is reversed by washing the muscle with saline.

Fig. 60. The top trace is an extracellular recording from a point half-way between the lip ganglion and cerebral ganglion of an external lip nerve; the bottom trace is an intracellular recording of the ipsilateral GSC. The preparation has been isolated from the animal for several hours, and constantly perfused with Meng's saline at room temperature (18-22°C). The GSC has been artificially stimulated to fire several thousand action potentials during the course of the experiment and is here fatigued. However the traces illustrate a bursting series which occurs synchronously in the GSC and in many other neurons which send axons into the external lip nerve. Approximately 20 sec before the onset of the bursts the resting potential of the GSC is decreased by some 5-10 mV (arrow). This presumably raises the potential of the GSC to threshold. Large epsp's (up to 15 mV and 1 sec duration) then occur in the GSC. At the same time as each epsp, a burst of spikes occurs in the external lip nerve. In this case the GSC did not actually spike, because the cell was fatigued. However at the start of the experiment 3 or 4 mV depolarization was sufficient to cause the GSC to fire spikes. Thus it would appear that should this phenomenon occur in the intact animal, the GSC would fire spikes synchronously with the other units in the external lip nerve. Such series of bursts were seen to occur once every 8 min in this preparation.

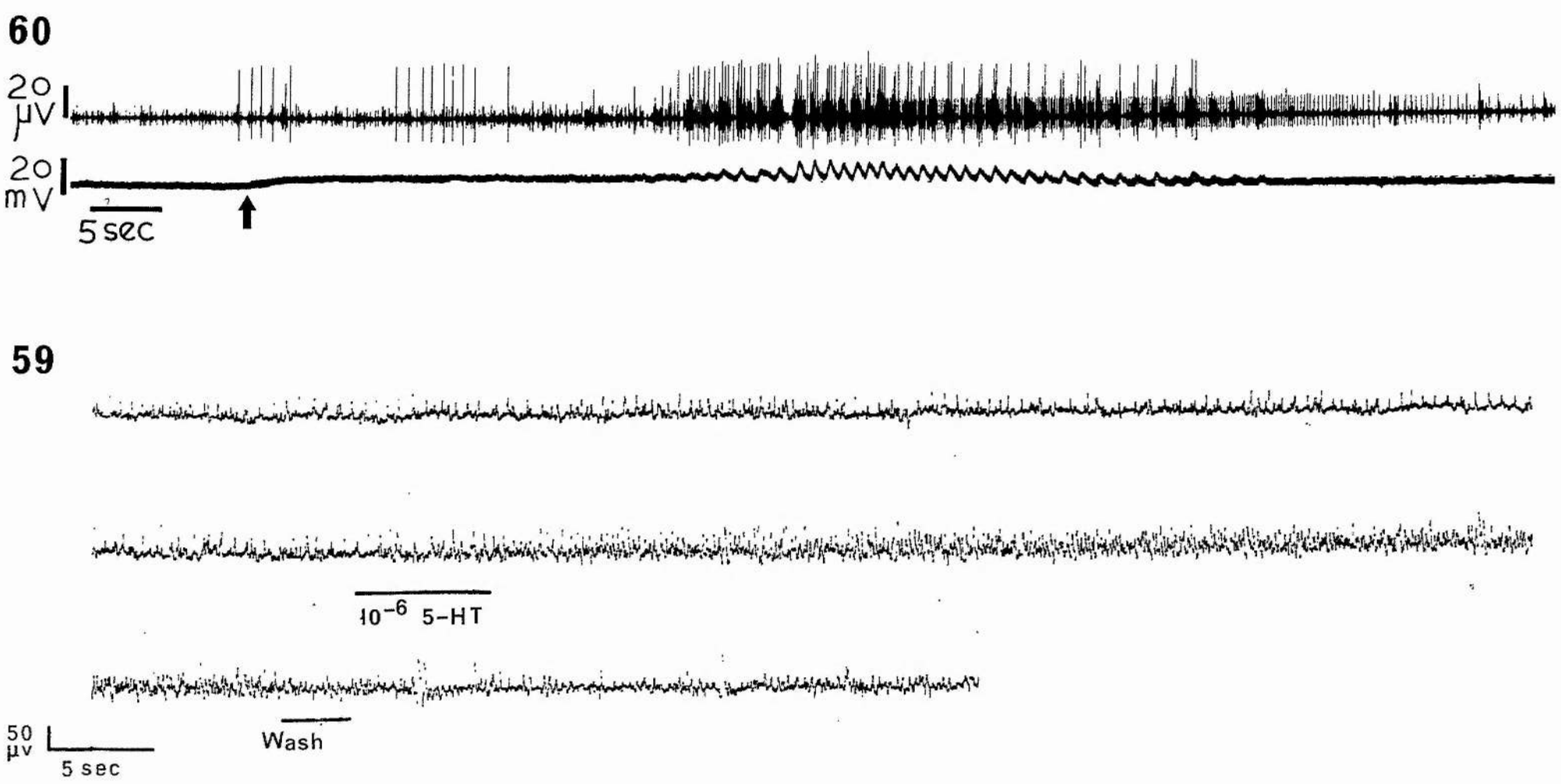

60
20 μV
20 mV
5 sec
59
10⁻⁶ 5-HT
50 μv
5 sec
Wash

Fig. 61. The top trace in A and B shows the degree of contraction (upwards) of a lip muscle recorded by attachment to a RCA 5734 transducer. Each bottom trace is an intracellular record from the ipsilateral GSC. In A, the muscle was made to contract maximally by direct stimulation with 3 V pulses each of 10 msec duration. These are seen as artefacts in the intracellular recording (between arrows). Following this the muscle was allowed to relax. In B, the same muscle was again made to contract maximally by direct stimulation (artefacts between arrows), but at the end of this stimulation the GSC was made to fire a burst of spikes by a direct depolarizing pulse. Following this the muscle relaxed more quickly. This effect was obtained with different lip musclesin several different preparations.

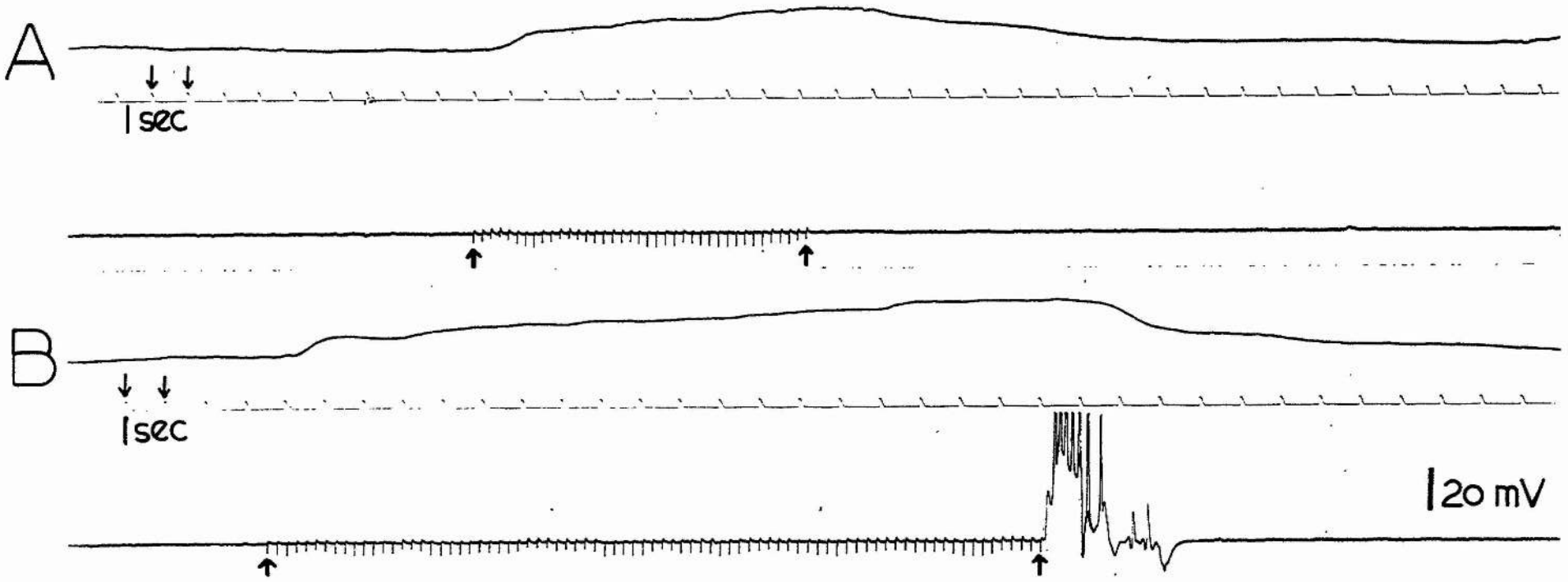
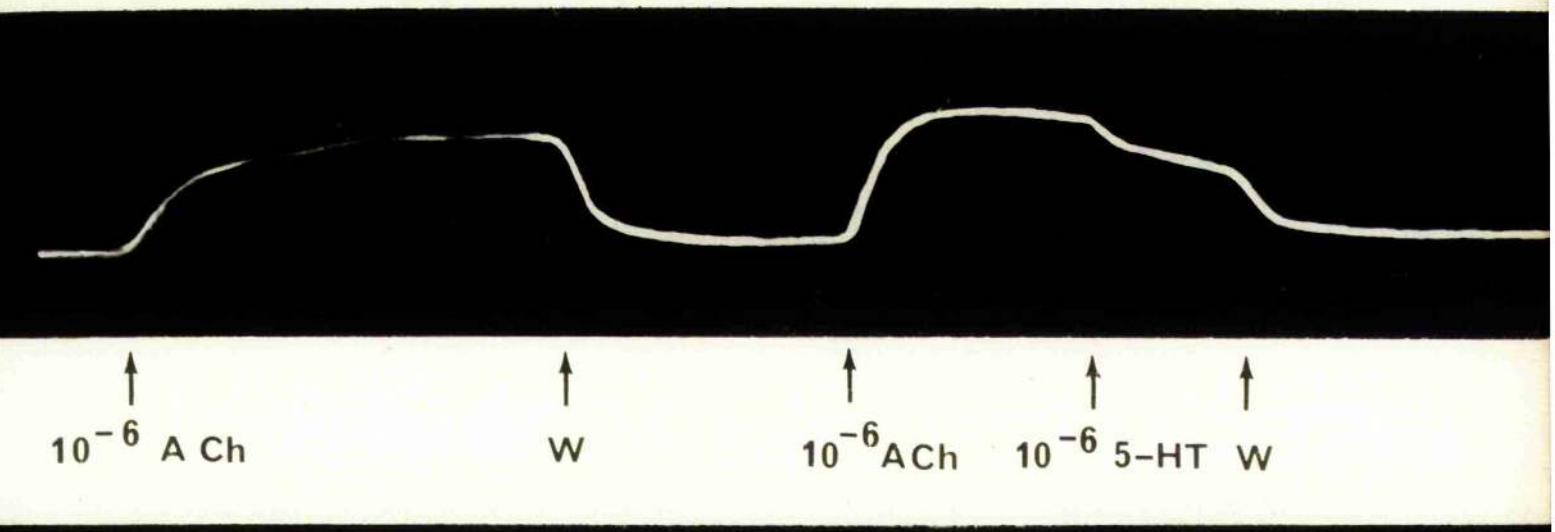

A
1 sec
B
1 sec
20 mV

Fig. 62. Kymograph record showing the changes in length of an individual lip muscle suspended in an organ bath when acetylcholine (ACh) and 5-HT were added to the bathing medium. Maximal contraction was produced by 10^{-6} g ACh/ml, and relaxation resulted when this was replaced with pure saline (W). The addition of 5-HT (bath concentration 10^{-6} g/ml) during an ACh-induced contraction resulted in partial relaxation. 5-HT had no effect on the length of the resting (relaxed) muscle.

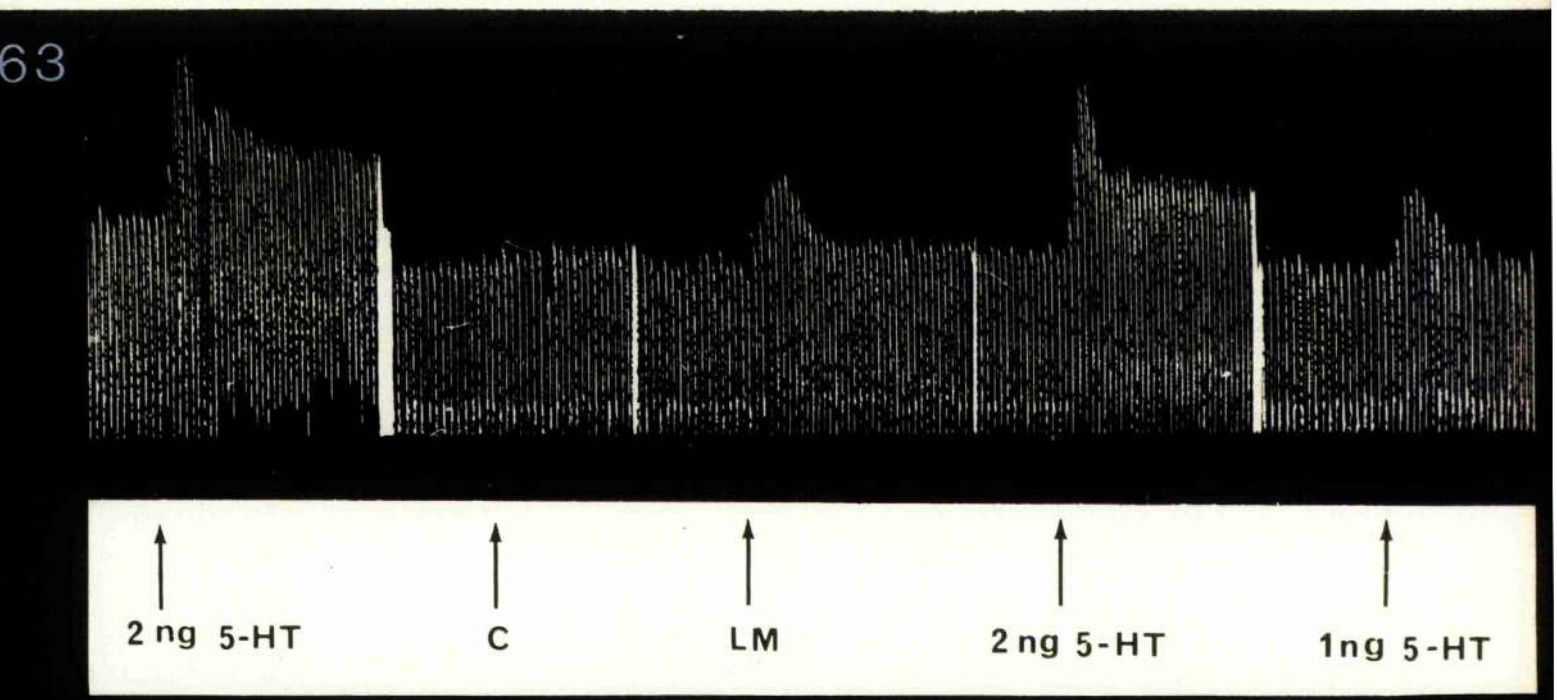
Fig. 63. Responses of an isolated heart of Helix aspersa to 5-HT, to an extract of lip muscles of H. pomatia prepared in Meng's saline (MM), and to an equivalent volume of saline taken from the bath of the dissected snail adjacent to the lip muscles (control, c). The amount of 5-HT in the lip muscle extract is approximately equivalent to 1 ng of authentic 5-HT. The results of this, and several other assays, indicate that the lip muscles contain approximately 0.1 µg 5-HT/g fresh tissue.

Fig. 64. Fluorescent micrograph of a section of lip musculature processed by the Falck technique (see Falck and Owman, 1965). The small branching nerve fluoresced a yellow to yellow-green colour, which indicates that some of the axons within the nerve contained 5-HT.

62



63



64

Fig. 65. The diagram in the middle shows, approximately to scale, the arrangement of the nerves leaving the buccal ganglia when viewed dorsally. The nomenclature of Schmalz (1914) has been adopted for the buccal nerves. The axon pathways taken by a right GSC is indicated by the dotted lines: The GSC axon branch in the cerebro-buccal connective (cbc) appears to divide into branches which run in the right second pharyngeal nerve (npr2), in the right posterior oesophageal nerve (opr), and across the buccal commissure. (npl2, second left pharyngeal nerve; npl3, third left pharyngeal nerve; npl1, first left pharyngeal nerve; sgl, left salivary gland nerve; oal, left anterior oesophageal nerve; opl, left posterior oesophageal nerve; npr3, third right pharyngeal nerve; oar, right anterior oesophageal nerve; sgr, right salivary gland nerve; nprl, first right pharyngeal nerve).

A. The top trace shows extracellular recordings from the right posterior oesophageal nerve in response to direct GSC stimulation (bottom trace). A small axon spike follows each GSC action potential. GSC stimulation also elicits firing of a large unit in the nerve. This large unit is probably involved in contractions of the oesophagus (M.S. Berry, personal communication).

B, C. The bottom trace in each record shows intracellular action potentials recorded in the GSC perikarya after direct stimulation with a depolarizing pulse; the top traces show spikes recorded from the anterior branch of the second pharyngeal nerve (B), and the posterior branch of the second pharyngeal nerve (C), resulting from such GSC stimulation. The spike recorded from the anterior nerve is smaller than that recorded from the posterior nerve, which indicates that the anterior nerve contains a smaller axon branch of the GSC than the posterior nerve. In relation to this the delay between the GSC action potential and the nerve spike is greater in B (approximately 50 msec, which indicates a conduction velocity in this GSC axon branch of approximately 20 cm/sec), than in C (approx. 40 msec, which indicates a conduction velocity of approx. 25 cm/sec). Time scale in each case is 40 msec. Lower voltage calibrations in each case is 20 mV. In B upper voltage calibration is 10 μV, in C upper voltage calibration is 20 μV.

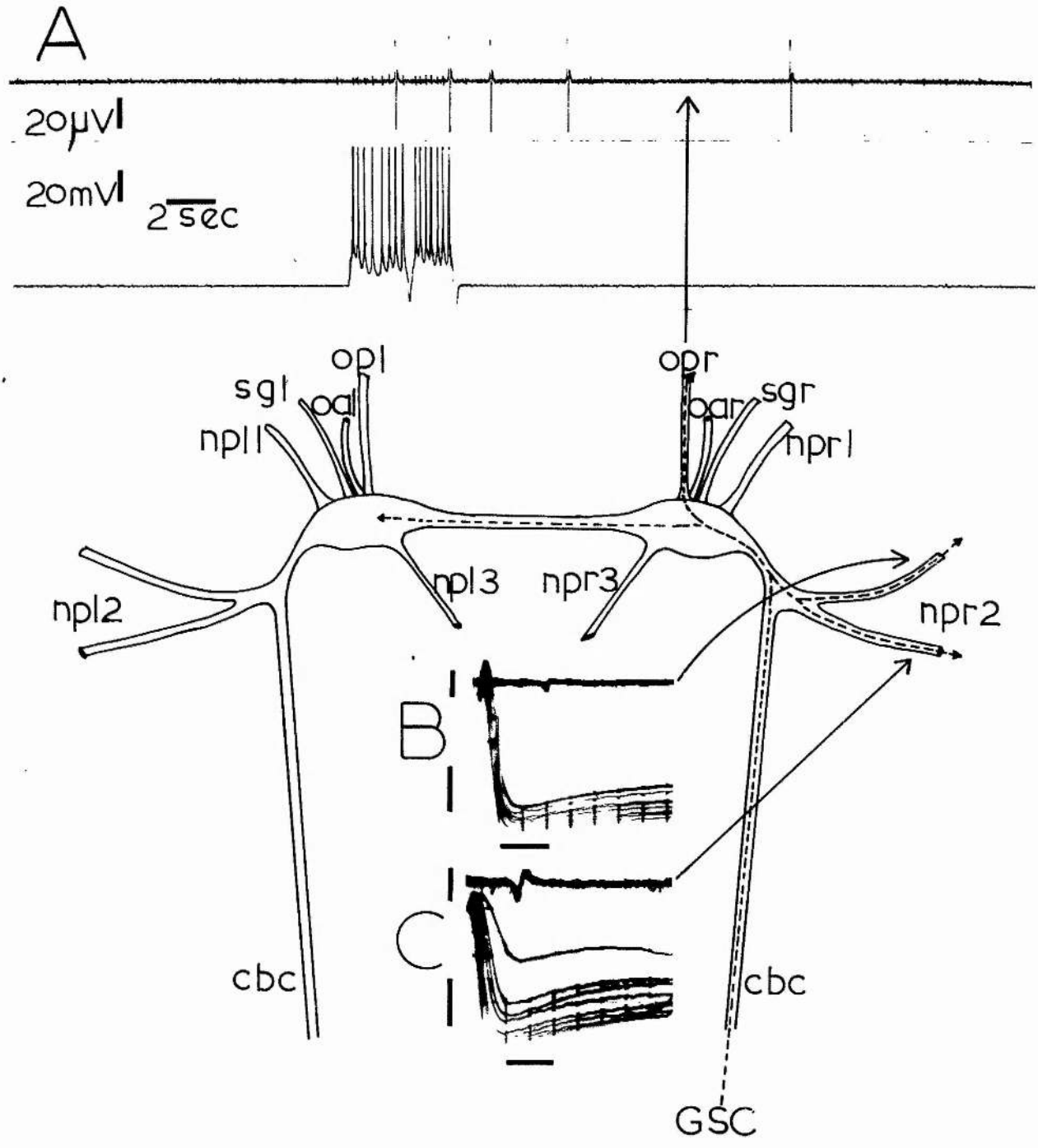

A
20μV
20mV
2 sec
opl
opr
sgl
oal
oar
sgr
npll
npr1
npl3
npr3
npl2
npr2
B
C
cbc
cbc
GSC

Fig. 66. Electron micrograph showing a part of a cross-section of the external lip nerve at a point half-way between the cerebral and lip ganglia. The area shown represents only a small proportion of the total cross-sectioned area of the nerve. The nerve is surrounded by connective tissue (c). Glial cells (one shown here, G) lie on the edge of the nerve, and send processes which ramify between axons. Not every axon is surrounded by glial cell processes, but groups of axons are (e.g. dotted lines). N, nucleus of glial cell.

The external lip nerve is an average-sized peripheral nerve of Helix pomatia. If every axon in the micrograph serves a specific function, then the total functional capacity of this typical nerve would seem alarmingly complex. On the other hand it is possible that many of the axons (which are presumably sensory and afferent in nature) serve similar functions; for example, each group which is effectively isolated anatomically by glial processes (such as that illustrated by the dotted lines) may serve one function.

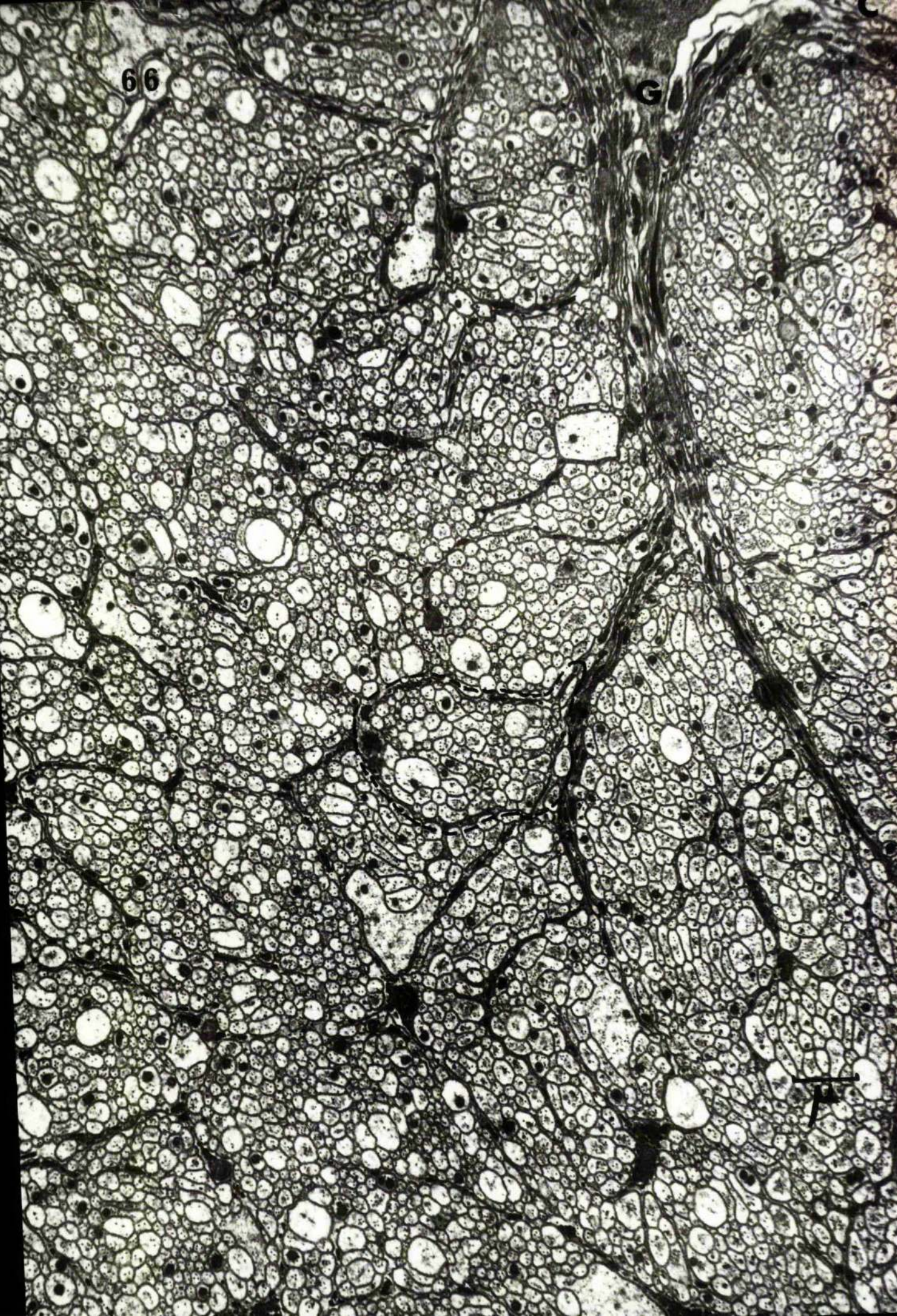
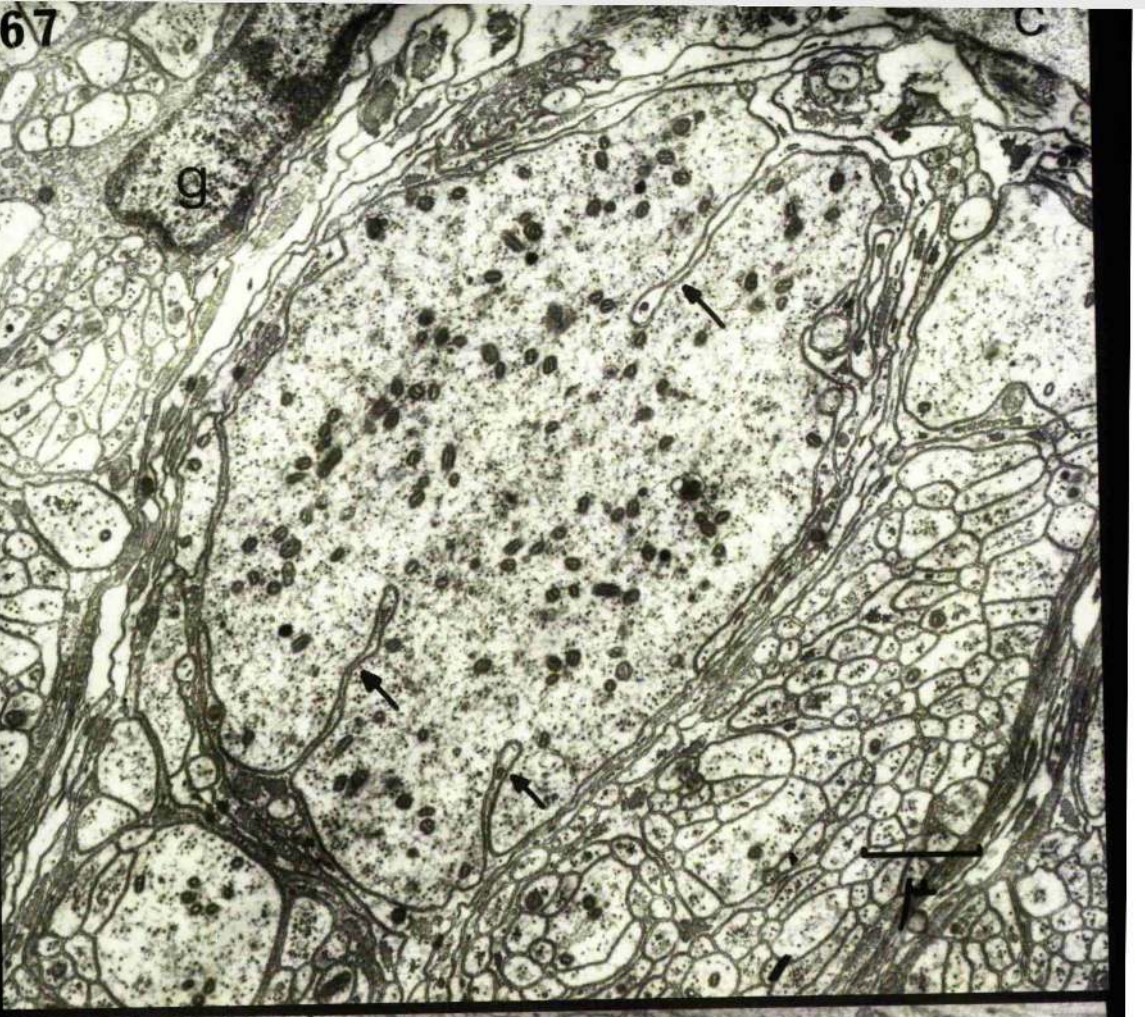
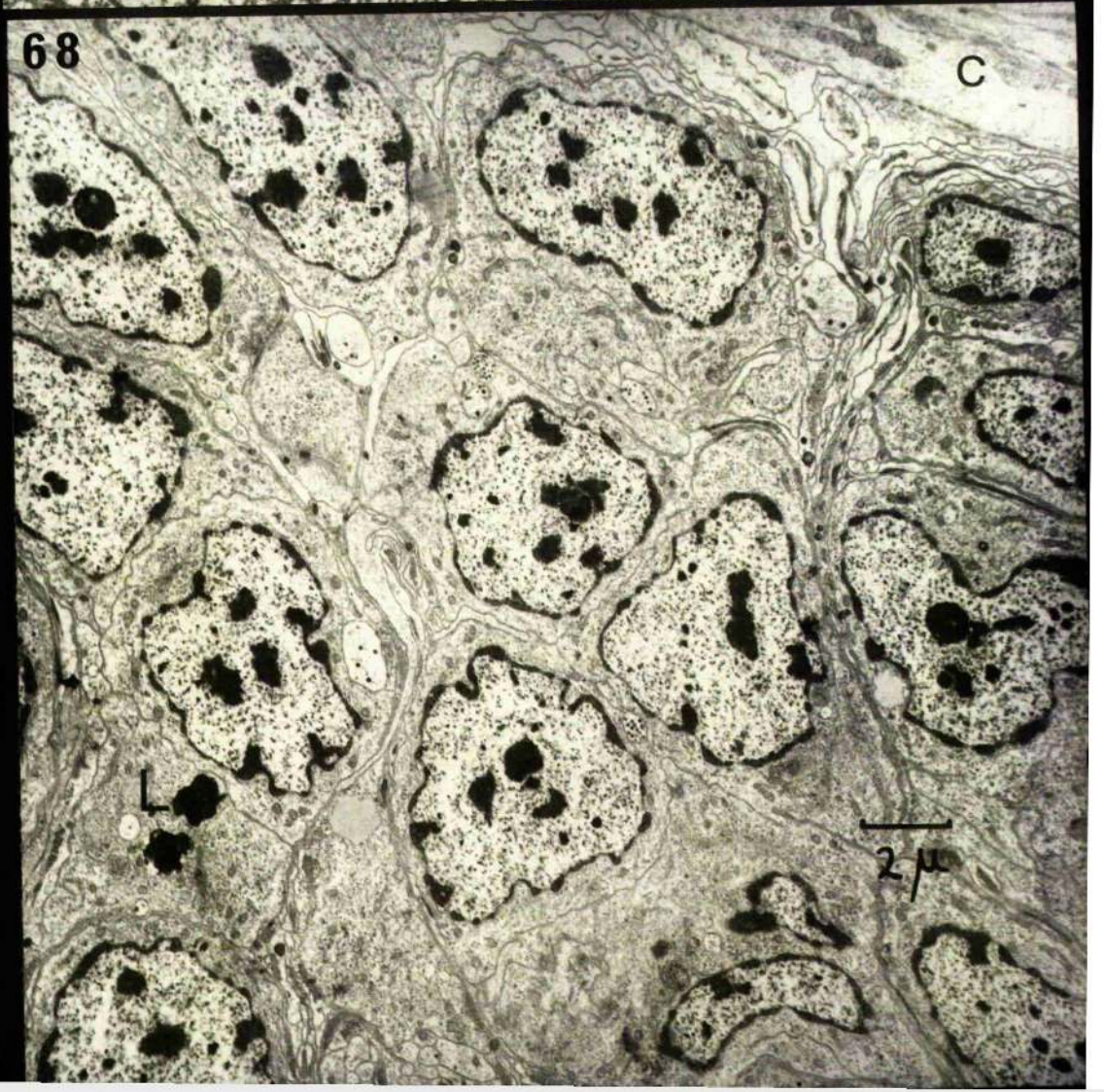

66
G

Fig. 67. Electron micrograph of a part of a cross-section of the external lip nerve of Helix pomatia showing one of the largest axons in the nerve. C, connective tissue sheath. G, glial cell nucleus. This particular axon contains numerous mitochondria and neurotubules. Glial cells processes penetrate the axon (arrows).

Fig. 68. Electron micrograph of a part of the outer edge of the ganglion situated at the peripheral end of the external lip nerve (see also Fig. 58). Beneath the connective tissue capsule (c) is a rind of small neuron perikarya, some of which are shown in the micrograph. L, lysosome-like bodies.


67
g
C
b


68
C
L
2μ

GENERAL DISCUSSION

5-HT was first isolated as enteramine from rabbit gastrointestinal mucosa and salivary glands of octopods by Erspamer and co-workers (see Erspamer, 1954), and as serotonin from serum by Rapport, Green and Page (1948). The substance was detected in the central nervous system of the dog by Amin, Crawford and Gaddum (1954). Since these studies much research has been undertaken to elucidate its role(s).

The substance is widely distributed in nature. It is present in plants, sponges, protozoa, most invertebrates, and all vertebrates that have been studied (for listings see Smith, 1971; Garattini and Valzelli, 1965; Welsh, 1968; Erspamer, 1966). Within vertebrate animals it occurs in the blood, the gastrointestinal tract, the skin, venoms, mast cells, the spleen, urine, the reproductive apparatus, and the central and peripheral nervous tissue (see Erspamer, 1966).

Much is known about the biochemistry of the substance in vertebrate tissues (see e.g. Blaschko and Levine, 1966). In particular much information is available on the regulation of its synthesis from the dietary precursor tryptophan (e.g. Hagen and Cohen, 1966; Wurtman and Fernstrom, 1972), and on its metabolism to the major end-product 5-hydroxyindoleacetic acid (Blaschko and Levine, 1966). The use of fluorescent histological methods (see Falck and Owman, 1965), has produced a full picture of the cellular localizations, and has shown especially the presence of serotonergic neurons and tracts within nervous systems. Electron microscopic techniques (Wood, 1965; Cannata, Chiocchio and Tramezzani, 1968) have provided evidence of the subcellular distributions of the amine.

The pharmacological effects of 5-HT are diverse. For example, the effects in mammals include complicated effects on blood pressure, respiration, and kidney functions (see Erspamer, 1966). In medicine, 5-HT levels have been observed to change in cancer (see Sandler, 1968), migraine (Curzon, 1968),

phenylketonuria (Pare, 1968), and certain other pathological conditions. However, 5-HT is no longer considered important in the pathogenesis of such diseases. 5-HT has been associated with certain behavioural and psychological responses, such as sleep (Jouvet, 1968), temperature regulation (Feldberg and Myers, 1964), sensory perception (see Way, 1972), and depression (Coppen, 1968).

The involvement of 5-HT in sleep and temperature regulation would appear especially interesting. These are described briefly in turn.

The 5-HT-containing neuron perikarya of the mammalian brain are almost exclusively located in the raphe nuclei (Dahlström and Fuxe, 1965). If brain 5-HT content is drastically reduced either by ablation of the raphe nuclei or by treatment with p-chlorophenylalanine, there is a marked decrease in time spent in slow-wave sleep (see Jouvet, 1968). The relative loss of sleep time appears to be proportional to the extent of depletion of 5-HT (Jouvet, 1968; Aprison and Hingten, 1972). Furthermore, after either MAO inhibition or intraventricular injection of 5-HT or 5-HTP, there appears to be an increased time spent in slow-wave sleep. On the other hand recent evidence (see Jouvet, 1972) suggests that catecholamine and acetylcholine-containing neurons are also important in the control of sleep. Thus it would appear that 5-HT-containing neurons are not exclusively important in sleep mechanisms.

Several pieces of evidence suggest that the 5-HT-containing neurons of the raphe also play some role in temperature regulation in mammals. Intraventricular injection of microgram quantities of 5-HT produces a marked elevation of body temperature (Feldberg and Myers, 1964), and the same effect is obtained following electrical stimulation of the raphe nuclei (see Weiss and Aghajanian, 1971). There is an increase in turnover of 5-HT in the raphe of the rat when the body temperature is raised artificially, which is accompanied by an increase in the firing rate of the raphe neurons (Weiss and Aghajanian, 1971). Changes in body temperature of the anaesthetized cat parallel spontaneous release of 5-HT from the presumed nerve endings of the raphe neurons (Holman and Vogt, 1972). However the mechanisms by which these changes are brought

about are far from clear. It is not known for example, whether the temperature elevating effect of 5-HT is due to a direct stimulatory effect on certain neurons, or whether it is due to inhibition of neurons which normally prevent temperature increase (see Cooper, Bloom, and Roth, 1970).

Despite the mass of information, much of which is relatively unambiguous (e.g. the data on localization, synthesis and turnover), it is perhaps disturbing that so little is known about the physiological function of 5-HT. After twenty years the evidence falls just short of proving one function amongst the many postulated, namely that 5-HT acts as a neurotransmitter.

Probably the greatest barrier to an understanding of its function is the lack of links which relate changes in behaviour to changes in physiological or biochemical activity. When 5-HT, or a drug which modifies, for example, the levels or effects of 5-HT, causes a pharmacological or behavioural change, it can be claimed that a link between behaviour and biochemistry has been shown; but the degree of precision of the link is difficult to evaluate. Thus in the mammalian central nervous system, direct and unambiguous effects are difficult to obtain because of a lack of specificity of action, rapid metabolism of the amine, and restrictions imposed by the blood-brain barrier (Shore, 1968). In fact, the possibility should be borne in mind that many pharmacological and behavioural effects of 5-HT may be quite unrelated to its normal functions. It would appear essential also to know, for example, what the mechanisms of synaptic transmission underlying the behavioural response are, or whether 5-HT is normally involved in the behaviour (Perry, 1968).

1. 5-HT as a neurotransmitter in mammals

The crucial evidence which shows that a particular substance is a neurotransmitter consists essentially of proving that, at the arrival of an action potential at the presynaptic ending, the substance is released in sufficient quantity to produce the physiological effect observed electrophysiologically in the postsynaptic structure (see Gerschenfeld, 1973). With the techniques

presently available this proof is unattainable, but it has been closely approached in the cases of acetylcholine in the neuromuscular junction of vertebrates, and noradrenaline in some peripheral adrenergic synapses. In other cases, such as that of 5-HT, proof is dependent upon the fulfilment of a variable number of indirect proofs (e.g. presence of the substance and enzymes for its synthesis in the presynaptic cell, release of the substance following nerve stimulation, mimicking of the effect of nerve ~~nerve~~ stimulation by the exogenously applied substance in all ways such as time course and conductance change, parallel action of drugs, and a mechanism(s) for removing the presumed transmitter substance following its release from presynaptic terminals). Various authors have compiled lists of such indirect criteria (e.g. Werman, 1966; Dudel, 1968; Gerschenfeld, 1966). In this way indirect evidence is accumulated until it becomes generally accepted that the substance is a likely transmitter. However, there is naturally disagreement about the amount of evidence necessary before such acceptance is made.

There is little disagreement, however, that there is at present insufficient evidence for 5-HT to be considered a neurotransmitter in the CNS of mammals. This lack of evidence reflects in part the difficulties in studying mammalian nervous tissue at the cellular level. Much of the available evidence is based upon the effects of drugs known to alter 5-HT metabolism, or which are structurally related to 5-HT. Unfortunately many of these investigations have had to rely on indirect and correlative effects of the various drugs, which are difficult to interpret.

Thus, for example, the data on the cellular effects of microelectrophoretically administered 5-HT in the mammalian brain are conflicting. Although in the majority of cases such administration has been found to decrease spontaneous activity (see Cooper, Bloom, and Roth, 1970), it is often impossible to rule out secondary effects. In some instances different workers have reported opposite effects caused by 5-HT on neurons of the same part of the brain of the same experimental animal (e.g. the lateral geniculate nucleus of the cat; see

Tōbecis and Di Maria, 1972). Furthermore no specific synaptic connection has yet been identified in the mammalian CNS which allows analysis of the effects of neurally released 5-HT compared with the effects of 5-HT administered by micropipettes. Because of this lack of information it is impossible to evaluate the multiple reports in the literature concerning the specificity of alleged 5-HT blocking agents which affect neuronal and behavioural activity, or whether the effects caused by exogenously applied 5-HT indicate the presence of excitatory or inhibitory 5-HT mediating synapses (Cooper, Bloom, and Roth, 1970).

On the other hand, there is fairly strong evidence indicating that 5-HT containing neurons play an integral role in the inhibitory control of gastric contraction in mammals (see Born, 1970; Gershon, 1970).

2. <u>The use of certain invertebrate nervous systems for studying neurotransmitters</u>

It appears that those substances found to date to be involved in chemical transmission, as well as the mechanism of their action on membranes, are the same both in vertebrates and invertebrates (see Gerschenfeld, 1973).

As mentioned above, studies on large masses of nervous tissue, such as parts of the mammalian CNS, which comprise many different types of neuron, as well as glial and other cell types, are sometimes difficult to interpret. This problem may be partially overcome by using tissues which are relatively rich in a population of neurons containing one type of transmitter (in the case of noradrenaline, for example, the adrenergic plexus in the iris; see Malmfors, 1965), or by using samples of parts of neurons (e.g. synaptosomes; Whittaker, 1964).

Another approach, and one which has been recently advocated by several workers (e.g. Hyden, 1967; Giacobini, 1969), is to study individual neurons of known transmitter type which can be readily and repeatedly identified in any animal of a particular species. Using this approach it is possible to obtain different information on different aspects of the neuron; for example, its

anatomy by dye injection and electron microscopy, its neurotransmitter content by micro-assay techniques, its biochemistry by micro-analytical techniques, and its electrical properties by intracellular electrophysiological techniques. As the information is collected it produces a picture of the function of the particular transmitter substance within the particular neuron. Furthermore these data, although small in relation to the total nervous complexity of the animal, can be compared with those obtained for other neurons, if for example, they contain the same neurotransmitter.

It would appear, therefore, that with the techniques presently available, the large, accessible and identifiable neurons of molluscs and some other invertebrates (e.g. those in the CNS of the leech, see Nicholls and Baylor, 1969; and in the CNS of some crustacea, see Wiersma, 1962, Kandel and Kupfermann, 1970) would offer certain unique advantages for studying many aspects of nerve biology. Much of our fundamental knowledge of the ionic mechanisms of nerve conduction has been obtained from cephalopod giant axons (Hodgkin and Huxley, 1952; Katz, 1966).

However, there is one problem in particular which is encountered in studies of individual neurons. This concerns the current use of giant neurons of gastropods for enzymatic and biochemical studies (e.g. Giller and Schwartz, 1971a, b; McCaman and Dewhurst, 1970; Weinreich, Dewhurst and McCaman, 1972; Coggeshall, Dewhurst, Weinreich and McCaman, 1972; Cottrell and Powell, 1971). One of the assumptions made in such studies is that any enzymatic activity found is due to the neuron itself, and not to adhering nerve terminals. This assumption is very probably correct, because no synapses have yet been seen with the electron microscope on the perikarya of giant neurons so far examined. However it is probably not correct to assume that such isolated neuron perikarya are free from glial cell contamination. Because glial cells have extensive infoldings into giant cell perikarya (see chapter 1) it is likely that they are isolated along with the neuronal perikarya. At present there are few data on the biochemical machinery of such glial cells. It would seem likely, however, that their biochemistry is not identical to that of the neuron which they cover. Such differences may

modify results of enzymatic studies made with the isolated nerve preparations. This problem should perhaps be more widely taken into consideration when drawing conclusions from biochemical results obtained with isolated neuron preparations.

3. Is 5-HT a neurotransmitter in gastropod molluscs?

It is perhaps evident from the data discussed in the present work that there are close parallels between the information available on the localization and biochemistry of 5-HT in molluscan and mammalian nervous tissue. However, there is very much more information on the biochemistry of 5-HT in the mammalian CNS than in molluscan nervous tissue (see e.g. Wurtman and Fernstrom, 1972; Glowinski, 1972), although new micromethods (e.g. Neuhoff, 1972) are adding rapidly to the knowledge in gastropods (e.g. Osborne, 1972a).

On the other hand, considerably more is known about the role of 5-HT in some neurons, and the nature of 5-HT receptors, in gastropods than in mammals (e.g. Cottrell and Laverack, 1968; Gerschenfeld, 1973). The recent advances made in these fields are evidence of the advantages of studying identifiable 5-HT--containing neurons. The results of the present work are consistent with a transmitter role for 5-HT, and have attempted to advance this supposition by illustrating certain features of serotonergic neurons of Helix pomatia. In particular they have provided evidence that an uptake mechanism of 5-HT and its immediate precursor is associated specifically with serotonergic neurons, that 5-HT may be located and transported intraneuronally within vesicles, and that 5-HT may be released from at least some nerve endings of a giant 5-HT-containing neuron onto muscles.

The writer is of the opinion that there is sufficient indirect, as well as direct evidence (see Cottrell, 1971a) that 5-HT serves a transmitter role in at least one situation; namely the OSCs of Helix pomatia. If further studies with other identified 5-HT-containing neurons produce similar results, it may soon be possible to accept 5-HT as a neurotransmitter in gastropod molluscs in general.

REFERENCES

ABBOT, N.J. Absence of blood-brain barrier in a Crustacean, Carcinus maenas L. Nature, 225, 291-293, (1970).

ABBOT, N.J. The organization of the cerebral ganglion in the shore crab, Carcinus maenas. II. The relation of intracerebral blood vessels to other brain elements. Z. Zellforsch. mikrosk. Anat., 120, 401-419, (1971).

ABBOT, N.J. Access of ferritin to the interstitial space of Carcinus from intracerebral blood vessels. Tissue and Cell, 4, 99-104, (1972).

AGHAJANIAN, G.K. Influence of drugs on the firing of serotonin-containing neurons in brain. Fed. Proc., 31, 91-97, (1972).

AGHAJANIAN, G.K. and BLOOM, F.E. Localization of tritiated serotonin in rat brain by electron-microscopic autoradiography. J. Pharmacol. exp. Therap., 156, 23-30, (1967).

AIRAKSINEN, M.M., GIACALONE, E. and VALZELLI, L. Hydroxylation of tryptophan in the brain in vivo. J. Neurochem., 15, 55-56, (1968).

AMIN, A.H., CRAWFORD, T.B.B. and GADDUM, J.H. The distribution of substance P and 5-hydroxytryptamine in the central nervous system of the dog. J. Physiol. (Lond.), 126, 596-618, (1954).

AMOROSO, E.C., BAXTER, M.I., CHIQUOINE, A.D. and NISBET, R.H. The fine structure of neurons and other elements in the nervous system of the giant African land snail Archachatina marginata. Proc. Roy. Soc. (B) 160, 167-180, (1964).

APRISON, M.H. and HINGTGEN, J.N. Serotonin and behavior: a brief summary. Fed. Proc., 31, 121-130, (1972).

ASCHER, P., GLOWINSKI, J., TAUC, L. and TAXI, J. Discussion of stimulation--induced release of serotonin. Adv. Pharmacol., 6A, 365-368, (1968).

ASHRENAZI, R., HOLMAN, R.B. and VOGT, M. Release of transmitters on stimulation of the nucleus linearis raphe in the cat. J. Physiol. (Lond.), 223, 255-259, (1972).

ATWOOD, H.L., HOYLE, G. and SMYTH, T. Mechanical and electrical properties of single innervated crab muscle fibres. J. Physiol., 180, 449-482, (1965).

BARDESSONO, F., GIACOBINI, E. and STEPITA-KLAUCO, M. Neuronal localisation of monoamines in the cerebral ganglia of the snail Helix pomatia. Brain Res., 47, 427-438, (1972).

BENJAMIN, P.R. and INGS, C.T. Golgi-Cox studies on the central nervous system of a gastropod mollusc. Z. Zellforsch. Mikros. Anat., 128, 564-582, (1972).

BENJAMIN, P.R. and PEAT, A. Myoneural junctions in the Connective tissue sheath of a Molluscan Ganglion. Nature, 219, 1371-1372, (1968).

BERN, H.A., NISHIOKA, R.S. and HAGADORN, I.R. Association of elementary neurosecretory granules with the Golgi complex. J. Ultrastruct. Res., 5, 311-320, (1961).

BERRY, M.S. A system of electrically coupled small cells in the buccal ganglia of the pond snail Planorbis corneus. J. Exp. Biol., 56, 621-637, (1972).

BERRY, M.S. and COTTRELL, G.A. Dopamine: Excitatory and inhibitory transmission from a giant dopamine neuron. Nature (In press).

BERTACCINI, G. Discussion. In: S.S. KETY and J. ELKES (Eds.) Regional Neurochemistry, p.305-306, London, Pergamon Press, (1961).

BLACKBURN, K.J., FRENCH, P.C. and MERRILLS, R.J. 5-hydroxytryptamine uptake by rat brain in vitro. Life Sci., 6, 1653-1663, (1967).

BLASCHKO, H. and HAWKINS, J. Observation on amine oxidase in cephalopods. J. Physiol., Lond., 116, 88-93, (1952).

BLASCHKO, H. and HOPE, D.B. Observations on the distribution of amine oxidase in invertebrates. Arch. Biochem. Biophys., 69, 10-15, (1957).

BLASCHKO, H. and LEVINE, W.G. Metabolism of indolealkylamines. In: "Handbook of Experimental Pharmacology" (O. EICHLER and A. FARCH, eds.) Vol. XIX, pp.212-244, Springer, New York, (1966).

BLOOM, F.E. and COSTA, E. The effects of drugs on serotonergic nerve terminals. In: Advances in Cytopharmacology. Ed. CLEMENTI, F. and CECCARELLI, B. pp.379-396, Raven Press, New York, (1971).

BLOOMQUIST, E. and CURTIS, B.A. The action of serotonin on calcium-45 efflux from the anterior byssal retractor muscle of Mytilus edulis. J. Gen. Physiol., 59, 476-485, (1972).

BOER, H.H. and LEVER, J. On the anatomy of the circulatory system in Ferrissia shimekii (Ancylidae, Pulmonata); especially on the blood supply of the central nervous system. Proc. Acad. Sci. Amst., (c), 62, 76-83 (1959).

BORN, G.V.R. 5-hydroxytryptamine receptors. In: Smooth Muscle. pp.418-450. Eds. BÜLBRING, E., BRADING, A.F., JONES, A.W. and TOMITA, T. Arnold Pub. Ltd., London. (1970).

BULLOCK, J.H. and HORRIDGE, G.A. Structure and function in the nervous systems of invertebrates. W.H. Freeman & Co., San Francisco and London. (1965).

CANNATA, M.A., CHIOCCHIO, S.R., TRAMEZZANI, J.H. Specificity of the glutaraldehyde silver technique for catecholamines and related compounds. Histochemie, 12, 253-265, (1968).

CARDOT, J. Decarboxylation in vitro du 5-hydroxytryptophane par le tissue nerveux du Mollusque Gastropode Helix pomatia. Comptes Rendus Acad. Sci. Paris, 256, 1036-1037, (1963).

CARDOT, J. Considérations sur le métabolisme de la 5-hydroxytryptamine et de la tryptamine chez le Mollusque Helix pomatia. Comptes Rendus Acad, Sci. Paris, 258, 1103-1105, (1964).

CARDOT, J. La monoamine oxidase chez le Mollusque Helix pomatia: activité sur quatre substrate. Comptes Rendus Soc. Biol., 160, 1264-1268, (1966).

CARDOT, J. Effets de la p-chlorophénylalamine sur les taux de 5-hydroxytryptamine des tissues nerveux et cardiaque chez le Mollusque Helix pomatia (L.). Comptes Rendus. 165, 97-100, (1971a).

CARDOT, J. Variations saisonnieses de la 5-hydroxytryptamine dans les tissue nerveux et cardiaque chez le Mollusque Helix pomatia. Comptes Rendus, 165, 338-341, (1971b).

CARDOT, J. Nouvelles observations sur l'innervation monoaminergique du coeur de deux Mollusques Gastropodes pulmonés: Helix pomatia et Limnea stagnalis. Comptes Rendus, 165, 1627-1630, (1971c).

CARDOT, J. Mise en évidence d'une activité tryptophane hydroxylasique dans le tissu nerveux du Mollusque Helix pomatia par utilisation in vitro de tryptophane 3-^{14}C. Résultats préliminaires. Comptes Rendus (D) 274, 1935-1938, (1972).

CARDOT, J. and HEROLD, J-P. Recherches histologiques et histochèmiques sur les celles granuleuses du coeur des mollusques Helix pomatia L. et lymnaea stagnalis L. Annal. Scient. Besançon., 7, 47-55, (1971).

CARDOT, J. and RIPPLINGER, J. Recherches sur les amines indoliques cardio-actives présentes dans le tissu nerveux du Mollusque Helix pomatia. J. Physiol. Paris, 55, 217-218, (1963).

CARLSSON, A., FUXE, K. and UNGERSTEDT, U. The effect of imipramine on central 5-hydroxytryptamine neurons. J. Pharm. Pharmacol., 20, 150-151, (1968).

CARO, L.G. and VAN TUBERGEN, R.P. High-Resolution Autoradiography. 1. Methods J. Cell Biol., 15, (1962), pp.173-188.

CARPENTER, D., BREESE, G., SCHANBERG, S. and KOPIN, I. Serotonin and dopamine: distribution and accumulation in Aplysia nervous and non-nervous tissues. Int. J. Neurosci., 2, 49-56, (1971).

CEDAR, H. and SCHWARTZ, J.H. Cyclic adenosine monophosphate in the nervous system of Aplysia californica. II. Effect of serotonin and dopamine. J. Gen. Physiol., 60, 570-587, (1972).

CEDAR, H., KANDEL, E.R. and SCHWARTZ, J.H. Cyclic adenosine monophosphate in the nervous system of Aplysia californica. I. Increased synthesis in response to synaptic stimulation. J. Gen. Physiol., 60, 558-569, (1972).

CHALAZONITIS, N. Chemopotentials in giant nerve cells (Aplysia fasciata). In: Nervous inhibition (E. FLOREY, Ed.) p.179-193. New York: Pergamon. (1961).

CHASE, T.N., BREESE, G.R., CARPENTER, D.O., SCHANBERG, S.M. and KOPIN, I.J. Stimulation-induced release of serotonin. Adv. Pharmacol., 6A, 351-364, (1968).

CHRISTENSON, J.G., DAIRMAN, W. and UDENFRIEND, S. On the identity of DOPA decarboxylase and 5-hydroxytryptophan decarboxylase. Nat. Acad. Sci. U.S.A., 69, 343-347, (1972).

COGGESHALL, R.E. A light and electron-microscope study of the abdominal ganglion of Aplysia californica. J. Neurophysiol., 30, 1263-1287, (1967).

COGGESHALL, R.E. A possible sensory-motor neuron in Aplysia californica. Tissue & Cell, 3, 637-649, (1971).

COGGESHALL, R.E. Autoradiographic and chemical localization of 5-hydroxytryptamine in identified neurons in the leech. Anat. Rec., 172, 489-498, (1972).

COGGESHALL, R.E., DEWHURST, H.A., WEINREICH, D.A. and McCAMAN, E. Aromatic acid decarboxylase and choline acetylase activities in a single identified 5-HT-containing cell of the leech. J. Neurobiol., 3, 259-266, (1972).

COLE, R.A. and TWAROG, B.M. Relaxation of catch is a molluscan smooth muscle I. Effects of drugs which act on the adenyl cyclase system. Comp. Biochem. Physiol. (A), 42, 321-330, (1972).

COOKE, I.M. The sites of action of pericardial organ extract and 5-hydroxytryptamine in the decapod crustacean heart. Am. Zoologist, 6, 107-121, (1966).

COOPER, J.R., BLOOM, F.E. and ROTH, R.H. The biochemical basis of neuropharmacology. Oxford Univ. Press, New York, (1970).

COPPEN, A.J. Depressed states and indolealkylamines. Adv. Pharmacol., 6B, 283-291, (1968).

CORRODI, H. and JONSSON, G. The formaldehyde fluorescence method for the histochemical demonstration of biogenic amines. J. Histochem. Cytochem., 15, 65-78, (1967).

CORRODI, H., HILLARP, N-Å. and JONSSON, G. Fluorescence methods for the histochemical demonstration of monoamines. 3. Sodium borohydride reduction of the fluorescent compounds as a specificity test. J. Histochem. Cytochem., 12, 582-586, (1964).

COTTRELL, G.A. Separation and properties of subcellular particles associated with 5-hydroxytryptamine and acetylcholine and with an unidentified cardio-excitatory substance from Mercenaria nervous tissue. Comp. Biochem. Physiol., 17, 893-907, (1966).

COTTRELL, G.A. Direct postsynaptic responses to stimulation of serotonin-containing neurons. Nature, 225, 1060-1062, (1970a).

COTTRELL, G.A. Actions of LSD-25 and reserpine on a serotonergic synapse. J. Physiol. (Lond.), 208, 28-29P., (1970b).

COTTRELL, G.A. Synaptic connections made by two serotonin-containing neurons in the snail (Helix pomatia) brain. Experientia, 27, 813-815, (1971a).

COTTRELL, G.A. Action of imipramine on 5-hydroxytryptaminergic transmission and on 5-hydroxytryptamine uptake in the snail (Helix pomatia) brain. Brit. J. Pharmacol., 43, 437P., (1971b).

COTTRELL, G.A. Action of imipramine on a serotonergic synapse. Comp. Gen. Pharmacol., 2, 125-128, (1971c).

COTTRELL, G.A. and LAVERACK, M.S. Invertebrate Pharmacology. Ann. Rev. Pharmacol., 8, p.273-298, (1968).

COTTRELL, G.A. and NASER, M. Subcellular localization of 5-hydroxytryptamine and substance X in molluscan ganglia. Comp. Biochem. Physiol., 20, p.901-906, (1967).

COTTRELL, G.A. and OSBORNE, N.N. Localization and mode of action of cardio-excitatory agents in molluscan hearts. Experientia, Suppl. 15, 220-231, (1969a).

COTTRELL, G.A. and OSBORNE, N.N. A neurosecretory system terminating in the Helix heart. Comp. Biochem. Physiol., 28, 1455-1459, (1969b).

COTTRELL, G.A. and OSBORNE, N.N. Subcellular localization of serotonin in an identified serotonin-containing neurone. Nature, 225, 470-472, (1970).

COTTRELL, G.A. and POWELL, B. Formation of serotonin by isolated serotonin-containing and by isolated non-amine-containing neurons. J. Neurochem., 18, 1695-1697, (1971).

COTTRELL, G.A., MACON, J. and SZCZEPANIAK, A.C. Glutamic acid mimicking of synaptic inhibition on the giant serotonin neurone of the snail. Brit. J. Pharmacol., 45, 684-688, (1972).

CURTIS, B.A. and BLOOMQUIST, E. Studies on the effects of serotonin (5-HT) and acetylcholine (ACh) on ^{45}Ca efflux from the anterior byssal retractor muscle. J. Gen. Physiol., 58, 706, (1971).

CURZON, G. 5-hydroxyindoles and migraine. Adv. Pharmacol., 6B, 191-200, (1968).

DAHL, E., FALCK, B., VON MECKLENBURG, C., MYHRBERG, H. and ROSENGREN, E. Neuronal localization of dopamine and 5-hydroxytryptamine in some molluscs. Z. Zellforsch., 71, 489-498, (1966).

DAHLSTRÖM, A. Axoplasmic transport (with particular respect to adrenergic neurons). Phil. Trans. Roy. Soc. London. B, 261, 325-358, (1971).

DAHLSTRÖM, A. and FUXE, K. Evidence for the existence of monoamine-containing neurone in the central nervous system. Acta Physiologica Scand., 62, Suppl. 232, 1-55, (1965).

DA PRADA, M. and PLETSCHER, A. Isolated 5-hydroxytryptamine organelles of rabbit blood platebits; physiological properties and drug induced changes. Brit. J. Pharmacol., 34, 591-597, (1968).

DE ROBERTIS, E., DE IRALDI, A.P., ARNAIZ, G.R. DE L. and SALGANICOFF, L. Cholinergic and noncholinergic nerve endings in rat brain. I. Isolation and subcellular distribution of acetylcholine and acetylcholine esterase. J. Neurochem., 9, 23-35, (1962).

DE VLIEGER, T.A. An experimental study of the tactile system of Lymnaea stagnalis (L.). Neth. J. Zool., 18, 105-154, (1968).

DIAZ, P.M., NAGI, S.H. and COSTA, E. Factors modulating brain serotonin turnover. Adv. Pharmacol., 6B, 75-92, (1968).

DUDEL, J. Facilitatory effects of 5-hydroxytryptamine on the crayfish neuromuscular junction. Naunyn-Schmiedebergs Arch. exp. Path. u. Pharmak., 249, 515-528, (1965).

DUDEL, J. Criteria for identification of transmitter substances. In: Structure and function of inhibitory neuronal mechanisms. Ed. VON EULER, C., SKOGLUND, S. and SÖDERBERG, U. pp.523-525, Pergamon Press, Oxford, (1968).

EALES, N.B. Aplysia. Proc. Trans. Liverpool Biol. Soc., 35, 183-266, (1921).

ECCLESTON, D., ASHCROFT, G.W. and CRAWFORD, T.B.B. 5-Hydroxyindole metabolism in rat brain. A study of intermediate metabolism using the technique of tryptophan loading - II. applications and drug studies. J. Neurochem., 12, 493-503, (1965).

ERSPAMER, V. Pharmacology of indolealkylamines. Pharmacol. Rev., 6, 425-487, (1954).

ERSPAMER, V. In "Handbook of Experimental Pharmacology" (O. EICHLER and A. FARCH, eds.) Vol. XIX, pp.132-181, 245-423, Springer, New York, (1966).

FALCK, B. Observations on the possibilities of the cellular localization of monoamines by a fluorescence method. Acta. Physiol. Scand., 56, Suppl. 197, (1962).

FALCK, B. and OWMAN, Ch. A detailed methodological description of the fluorescence method for the cellular demonstration of biogenic monoamines. Acta Univ. Lund., Sect. II. NO.7, 1-23, (1965).

FÄNGE, R. and MATTISSON, A. Studies on the physiology of the radula muscle of Buccinum undatum. Acta zool., 39, 53-54, (1958).

FELDBERG, W. and MYERS, R.D. Effects on temperature of amines injected into the cerebral ventricles. A new concept of temperature regulation. J. Physiol. 173, 226-237, (1964).

FERNANDEZ, J. Nervous system of the snail Helix aspersa. I. Structure and histochemistry of ganglionic sheath and neuroglia. J. comp. Neurol. 127, 157-182, (1966).

FERNANDEZ, J. Nervous system of the snail Helix aspersa. II. Fine structure of vascular channels and amebocytes associated with the ganglionic sheath. Z. Zellforsch., 118, 512-524, (1971).

FERNANDEZ, J. and FERNANDEZ, M.S. Nervous system of the snail Helix aspersa. III. Electron microscopic study of neurosecretory nerves and endings in the ganglionic sheath. Z. Zellforsch., 135, 473-482, (1972).

FERNSTROM, J.D. and WURTMAN, R.J. Brain serotonin content: Physiological regulation by plasma neutral amino acids. Science, 178, 414-416, (1972).

FLOREY, E. Comparative pharmacology: Neurotropic and myotropic compounds. Ann. Rev. Pharmacol., 5, 357-381, (1965).

FLOREY, E. Neurotransmitters and modulators in the animal kingdom. Fed. Proc., 26, 1164-1178, (1967).

FRAZIER, W.T., KANDEL, E.R. KUPFERMANN, I., WAZIRI, R. and COGGESHALL, R.E. Morphological and functional properties of identified neurons in the abdominal ganglion of Aplysia californica. J. Neurophysiol., 30, 1288-1351, (1967).

GAL, E.M., ARMSTRONG, J.C. and GINSBERG, B. The nature of in vitro hydroxylation of L-tryptophan by brain tissue. J. Neurochem., 13, 643-654, (1966).

GAL, E.M., POCZIK, M. and MARSHALL, F.D. Hydroxylation of tryptophan to 5-hydroxytryptophan by brain tissue in vitro. Biochem. Biophys. Res. Commun., 12, 39-43, (1963).

GARATTINI, S. and VALZELLI, L. Serotonin. Elsevier, Amsterdam, (1965).

GERSCHENFELD, H.M. Observations in the ultrastructure of synapses in some pulmonate molluscs. Z. Zellforsch. Mikrosh. Anat., 60, 258-275, (1963).

GERSCHENFELD, H.M. Chemical transmitters in invertebrate nervous systems. Symp. Soc. Exp. Biol., 20, 299-323, (1966).

GERSCHENFELD, H.M. Serotonin: two different inhibitory actions on snail neurones. Science, 171, 1252-1254, (1971).

GERSCHENFELD, H.M. Chemical transmission in invertebrate central nervous system and neuromuscular junctions. Physiol. Rev., 53, 1-119, (1973).

GERSCHENFELD, H.M. and STEFANI, E. An electrophysiological study of 5-hydroxytryptamine receptors of neurones in the molluscan nervous system. J. Physiol., 185, 684-700, (1966).

GERSCHENFELD, H.M. and STEFANI, E. Evidence for an excitatory transmitter role of serotonin in molluscan central synapses. Adv. in Pharmacol., 6A, 369-392, (1968).

GERSCHENFELD, H.M. and TAUC, L. Pharmacological specificities of neurones in an elementary nervous system. Nature, 189, 924-925, (1961).

GERSCHENFELD, H.M. and TAUC, L. Différent aspects de la pharmacologie des synapses dans le système nerveux central des Mollusques. J. Physiol. (Paris), 56, 360-361, (1964).

GERSCHENFELD, H.M., ASCHER, P. and TAUC, L. Two different excitatory transmitters acting on a single molluscan neurone. Nature, 213, 358-359, (1967).

GERSCHON, M.D. The identification of neurotransmitters to smooth muscle. In: Smooth Muscle. pp.496-524. Eds. BÜLBRING, E., BRADING, A.F., JONES, A.W. and TOMITA, T. Arnold Pub. Ltd., London, (1970).

GERSCHON, M.D. and ROSS, L.L. Radioisotopic studies of the binding exchange, and distribution of 5-hydroxytryptamine synthesized from its radioactive precursor. J. Physiol., 186, 451-476, (1966).

GIACOBINI, E. Chemistry of isolated invertebrate neurons. In: Handbook of Neurochemistry, 2, 195-239, Plenum, New York, (1969).

GILLER, E. and SCHWARTZ, J.H. Choline acetyltransferase in identified neurons of abdominal ganglion of Aplysia californica. J. Neurophysiol., 34, 93-107, (1971a).

GILLER, E. and SCHWARTZ, J.H. Acetylcholinesterase in identified neurons of abdominal ganglion of Aplysia californica. J. Neurophysiol., 34, 108-115, (1971b).

GLAIZNER, B. Pharmacological mapping of cells in the suboesophageal ganglia of Helix aspersa. In: Symposium in Neurobiology of Invertebrates. Budapest, Akadêmiac Kiadó, pp.267-284, (1967).

GLOWINSKI, J. Some new facts about synthesis, storage, and release processes of monoamines in the central nervous system. In: Perspectives in Neuropharmacology. Ed. SNYDER, S.H. pp.349-403. Oxford Univ. Press, (1972).

GORMAN, A.L.F. and MIROLLI, M. Axonal localization of an excitatory post-synaptic potential in a molluscan neurone. J. Exp. Biol., 53, 727-736, (1970).

GRAHAME-SMITH, D.G. The enzymic conversion of tryptophan into 5-hydroxytryptophan by isolated brain tissue. Biochem. J., 92, 52P., (1964a).

GRAHAME-SMITH, D.G. Tryptophan hydroxylation in brain. Biochem. Biophys. Res. Commun., 16, 586-592, (1964b).

GRAHAME-SMITH, D.G. The Biosynthesis of 5-Hydroxytryptamine in Brain. Biochem. J., 105, 351-359, (1967).

GRAHAME-SMITH, D.G. Studies in vivo on the relationship between brain tryptophan, brain 5-HT synthesis and hyperactivity in rats treated with a monoamine oxidase inhibitor and L-tryptophan. J. Neurochem., 18, 1053-1066, (1971).

GRAHAME-SMITH, D.G. and PARFITT, A.G., Tryptophan transport across the synaptosomal membrane. J. Neurochem., 17, 1339-1353, (1970).

GRAY, E.G. The fine structural characterization of different types of synapses. Prog. in Brain Res., 34, 149-160, (1971).

GREEN, H. and SAWYER, J.L. Demonstration, characterization, and assay procedure of tryptophan hydroxylase in rat brain. Analyt. Biochem., 15, 33-42, (1966).

GRUNDFEST, H., REUBEN, J.P. and RICKLES, W.H. The electrophysiology and pharmacology of lobster neuromuscular synapses. J. Gen. Physiol., 42, 1301-1323, (1959).

GUBICZA, A. and S.-RÓZSA, K. Identification of central neurons innervating the heart of Lymnaea stagnalis L. (Gastropoda). Annal. Biol. Tihany., 36, 3-10, (1969).

HAGEN, P.B. and COHEN, L.H. Biosynthesis of indolealkylamines. Physiological release and transport of 5-hydroxytryptamine. In "Handbook of Experimental Pharmacology" (O. EICHLER and A. FARAH, eds.). Vol. XIX, p.182-211, Springer, New York, (1966).

HAGIWARA, S. and MORITA, H., Electrotonic transmission between two nerve cells in leech ganglion. J. Neurophysiol., 25, 721-731, (1962).

HENN, F.A. and HAMBERGER, A. Glial cell function: Uptake of transmitter substances. Proc. Nat. Acad. Sci. U.S.A., 68, 2686-2690, (1971).

HÉRY, F., ROUER, E. and GLOWINSKI, J. Daily variations of serotonin metabolism in the rat brain. Brain Res., 43, 445-465, (1972).

HIDAKA, T., OSA, T., and TWAROG, B.M. The action of 5-hydroxytryptamine on Mytilus smooth muscle. J. Physiol., 192, p.869-877, (1967).

HILL, R.B. The effects of certain neurohumors and of other drugs on the ventricle and radula protractor of Busycon canaliculatum and on the ventricle of Strombus gigas. Biol. Bull., Woods Hole, 115, 471-482, (1958).

HILL, R.B. and WELSH, J.H. Heart, Circulation and Blood Cells. In: Physiology of Mollusca, II. Eds. WILBUR, K.M. and YONGE, C.M., pp.125-174, Academic Press, New York and London, (1966).

HILLE, B. Ionic channels in nerve membranes. In: Progress in Biophysics. Ed. BUTLER, J.A.V. and NOBLE, D., 21, pp.3-32, Pergamon Press, (1970).

HIRIPI, L. and SALANKI, J. 5-HTP-Dopa decarboxylase in the nervous system and other tissues of Anodanta cygnea L. (Pelecypoda). Ann. Biol. Tihany, 36, 19-24, (1969).

HODGKIN, A.L. and HUXLEY, A.F. A quantitative description of membrane current and its application to conduction and excitation in nerve. J. Physiol., 117, 500-544, (1952).

HOLMAN, R.B. and VOGT, M. Release of 5-hydroxytryptamine from caudate nucleus and septum. J. Physiol. (Lond.), 223, 243-254, (1972).

HOLMGREN, E. Beitrage zur Morphologie der Zelle. I. Nervenzellen. Anat. Hefte., 18, 269-325, (1901).

HUGHES, G.M. and TAUC, L. The path of the giant cell axons in Aplysia depilans. Nature, 191, 404-405, (1961).

HYDEN, H. Dynamic aspects on the neuron - glia relationship. A study with micro-chemical methods. In: The Neuron. Ed. HYDEN, H. pp.179-219, Elsevier, Amsterdam, (1967).

ICHIYAMA, A., NAKAMURA, S., NISHIZUKA, Y. and HAYAISHI, O. Enzymic studies on the biosynthesis of serotonin in mammalian brain. J. Biol. Chem., 245, 1699-1709, (1970).

IVERSEN, L.L. Neuronal uptake process for amines and amino-acids. In: Biochemistry of Simple Neuronal Models. Ed. COSTA, E. and GIACOBINI, E., New York. Raven Press, (1970).

IVERSEN, L.L. Role of transmitter uptake mechanisms in synaptic neurotransmission. Br. J. Pharmacol., 41, 571-591, (1971).

JAEGER, C.P. Neuroendocrine regulation of cardiac activity in the snail Strophocheilus oblongus. Comp. Biochem. Physiol., 17, 409-415, (1966).

JAEGER, C.P., JAEGER, E.C. and WELSH, J.H. Localization of monoamine-containing neurones in the nervous system of Strophocheilus oblongus (Gastropoda). Z. Zellforsch. mikrosk. Anat., 112, 54-68, (1971).

JAIM-ETCHEVERRY, G. and ZIEHER, L.M. Ultrastructural cytochemistry and pharmacology of 5-hydroxytryptamine in adrenergic nerve endings. I. Localization of exogenous 5-hydroxytryptamine in the autonomic nerves of the rat vas deferens. J. Pharm. Exptl. Therap., 166, 264-271, (1969).

JEQUIR, E., LOVENBERG, W. and SJOERDSMA, A. Tryptophan hydroxylase inhibition: the mechanism by which p-chlorophenylalanine depletes rat brain serotonin. Molec. Pharmacol., 3, 274-278, (1967).

JOURDAN, F. and NICAISE, G. Cytochimie ultrastructurale de la serotonine dans le système nerveux central de l'Aplysie. Proc. 7th Int. Congr. Electron Micros. Grenoble, pp.677-678, (1970).

JOUVET, M. Insomnia and decrease of cerebral 5-hydroxytryptamine after destruction of the raphe system in the cat. Adv. Pharmacol., 6B, 265-279, (1968).

JOUVET, M. The role of monoamines and acetylcholine containing neurons in the regulation of the sleep waking cycle. Ergebnisse der Physiologie, 64, 166-307, (1972).

JUORIO, A.V. and KILLICK, S.W. The effect of drugs on the synthesis and storage of monoamines in nervous tissue of molluscs. Int. J. Neurosci., 4, 195-202, (1972a).

JUORIO, A.V. and KILLICK, S.W. Monoamines and their metabolism in some molluscs. Comp. Gen. Pharmacol., 3, 283-295, (1972b).

KANDEL, E.R. and KUPFERMANN, I. The functional organization of invertebrate ganglia. Ann. Rev. Physiol., 32, 193-258, (1970).

KANDEL, E.R. and TAUC, L. Input organization of two symmetrical giant cells in the snail brain. J. Physiol., 183, 269-286, (1966a).

KANDEL, E.R. and TAUC, L. Anomalous rectification in the metacerebral giant cells and its consequences for synaptic transmission. J. Physiol. (Lond.), 183, 287-304, (1966b).

KAROBATH, M. Serotonin synthesis with rat brain synaptosomes. Effects of serotonin and monoamineoxidase inhibitors. Biochem. Pharmacol., 21, 1253-1265, (1972).

KATZ, B. Nerve, Muscle and Synapse. McGraw - Hill, (1966).

KERKUT, G.A. and COTTRELL, G.A. Acetylcholine and 5-hydroxytryptamine in the snail brain. Comp. Biochem. Physiol., 8, 53-63, (1963).

KERKUT, G.A. and LAVERACK, M.S. A cardio-accelerator present in tissue extracts of the snail Helix aspersa. Comp. Biochem. Physiol., 1, 62-71, (1960).

KERKUT, G.A. and WALKER, R.J. The effects of drugs on the neurones of the snail, Helix aspersa. Comp. Biochem. Physiol., 3, 143-160, (1961).

KERKUT, G.A. and WALKER, R.J. The specific chemical sensitivity of Helix nerve cells. Comp. Biochem. Physiol., 7, 277-288, (1962).

KERKUT, G.A., SEDDEN, G.B. and WALKER, R.J. Uptake of DOPA and 5-hydroxy-tryptophan by monoamine forming neurones in the brain of Helix aspersa. Comp. Biochem. Physiol., 23, 159-162, (1967).

KNOTT, P.J. and CURZON, G. Free tryptophan in plasma and brain tryptophan metabolism. Nature, 239, 452-453, (1972).

KRISHNA, G., WEISS, B., DAVIES, J. and HYMIE, S. Mechanism of nicotinic acid inhibition of hormone-induced Lipolysis. Fed. Proc. Fedn. Am. Socs. exp. Biol., 25, 719, (1966).

KRISHNA, G., FORN, J., VOIGHT, K., PAUL, M. and GESSA, G.L. Dynamic aspects of neurohormone control of cyclic 3', 5'-AMP synthesis in brain. In: Role of Cyclic AMP in Cell Function. Eds. GREENGARD, P. and COSTA, E. pp.155-186, Raven Press, New York, (1970).

KUNZE, H. Zur Topographie und Histologie des Centralnerven-systems von Helix pomatia L. Z. Wiss. Zool., 116, 25-203, (1921).

LENT, C.M. A comparative study of the neuronal geometry of Retzius' cells in leeches. Am. Zoologist, 11, 675, (1971).

LENT, C.M. Electrophysiology of Retzius' cells of segmental ganglion in the horse leech, Haemopis marmorata (Say). Comp. Biochem. Physiol., A, 42,

LENT, C.M. Retzius cells: Neuroeffectors controlling mucus release by the leech. Science, 179, 693-696, (1973).

LOEWI, O. Über humorale Übertagbarbeit der Herznouenwirkung. Pflügers Arch. ges. Physiol., 189, 239-242, (1921).

LOVELAND, R.E. 5-Hydroxytryptamine, the probable mediator of excitation in the heart of Mercenaria (Venus) Mercenaria. Comp. Biochem. Physiol., 9, 95-104, (1963).

LOVENBERG, W., JEQUIER, E. and SJOERDSMA, A. Tryptophan hydroxylation: Measurement in pineal gland, brain stem and carcinoid tumor. Science, 155, 217-219, (1967).

LOVENBERG, W., JEQUIER, E. and SJOERDSMA, A. Tryptophan hydroxylation in mammalian systems. Adv. Pharmacol., 6A, 21-36, (1968).

MACKAY, A.R. and GELPERIN, A. Pharmacology and reflex responsiveness of the heart in the giant garden slug, Limax maximus. Comp. Biochem. Physiol., A, 43, 877-896, (1972).

MACON, J.B., SOKOLOFF, L. and GLOWINSKI, J. Feedback control of rat brain 5-hydroxytryptamine synthesis. J. Neurochem., 18, 323-331, (1971).

MALMFORS, T. Studies on adrenergic nerves. The use of rat and mouse iris for direct observations on their physiology and pharmacology at cellular and subcellular levels. Acta. Physiol. Scand., 64, Suppl. 248, pp.1-93, (1965).

MANSOUR, T.E. Factors influencing activation of phosphofructokinase. Pharmac. Rev., 18, 173-179, (1966).

MARGOLIS, R.J. and LAJTHA, A. Ion dependence of amino acid uptake in brain slices. Biochem. Biophys. Acta., 163, 373-385, (1968).

MARSDEN, C.A. The occurrence of 5-hydroxyindoleacetic acid in the central nervous system of Planorbis corneus. Comp. Gen. Pharmacol., 3, 1-5, (1972).

MARSDEN, C.A. and KERKUT, G.A. Fluorescence microscopy of the 5-HT and catecholamine containing cells in the central nervous system of the leech, Hirudo medicinalis. Comp. Biochem. Phsyiol., 31, 851-862, (1969).

MARSDEN, C.A. and KERKUT, G.A. The occurrence of monoamines in Planorbis corneus: a fluorescence microscopic and microspectrometric study. Comp. Gen. Pharmacol., 1, 101-116, (1970).

McCAMAN, R.E. and DENHURST, S.A. Choline acetyltransferase in individual neurons of Aplysia californica. J. Neurochem., 17, 1421-1428, (1970).

MENG, K. Untersuchungen zur Störung der Herztätigkeit beim Helix pomatia. Zool. Jber Neapel 68, 539-566, (1960).

MICHAELSON, I.A. and WITTAKER, V.P. The subcellular localization of 5-hydroxytryptamine in guinea pig brain. Biochem. Pharmacol., 12, 203-211, (1963).

MINIHAN, K. and DAVIES, R.E. Energy requirements for relaxation from tonic contractions ("catch") in invertebrate muscle. Nature, 208, 1327-1329, (1965).

MIROLLI, M. Discussion of evidence for an excitatory transmitter role of serotonin in molluscan central synapses. Adv. in Pharmacol., 6A, 393-394, (1968).

MIROLLI, M. and WELSH, J.H. The effects of reserpine and LSD on molluscs. In: Comparative Neurochemistry. Ed. RICHTER, D. pp.433-443, Pergamon Press, (1964).

MOIR, A.T.B. and ECCLESTON, D. The effects of precursor loading in the cerebral metabolism of 5-hydroxyindoles. J. Neurochem., 15, 1093-1108, (1968).

NEFF, N.H. and TOZER, T.N. In vivo measurement of brain serotonin turnover. Adv. Pharmacol., 6A, 97-109, (1968).

NEUHOFF, V. Neurochemical micromethods. Int. J. Neuroscience, 4, 93-101, (1972).

NEUHOFF, V. and WEISE, M. Determination of picomole quantities of γ- amino butyric-acid (GABA) and serotonin. Arzneim - Forsch. (Drug Res.), 20, 368-372, (1970).

NICAISE, G., PAVANS DE CECCATTY, M., and BAILEYDIER, C. Ultrastructures des connexions entre cellules nerveuses, musculaires, et glio-interstitielles chez Glossodoris. Z. Zellforsch., 88, 470-486, (1968).

NICHOLLS, J. and BAYLOR, D.A. The specificity and functional role of individual cells in a simple central nervous system. Endeavor, 28, 3-7, (1969).

NOLD, R. Die Histologie des Blutgefäßsystems und des Herzens von Helix pomatia. Z. wiss. Zool., 123, 373-430, (1924).

NOLTE, A. The mode of release of neurosecretory material in the freshwater pulmonate Lymnaea stagnalis L. (Gastropoda). Symp. Neurobiol. Inverts., 123-133, (1967).

NOLTE, A., BREUCKER, H. and KUHLMANN, D. Cytosomale Einschlüsse und Neurosekret im Nervengewebe von Gastropoden. Z. Zellforsch. Mikrosk. Anat., 68, 1-27, (1965)

OSBORNE, N.N. Distribution, localization and functional significance of biologically active monoamines in gastropod molluscs. Ph.D. Thesis. St.Andrews University, Scotland.

OSBORNE, N.N. The *in vivo* synthesis of serotonin in an identified serotonin-containing neuron of *Helix pomatia*. Internat. J. Neuroscience, 3, 215-228, (1972a).

OSBORNE, N.N. Effect of electrical stimulation on the *in vivo* metabolism of glucose and glutamic acid in an identified neuron. Brain Res., 41, 237-241, (1972b).

OSBORNE, N.N. Serotonin, free amino-acids, and related substances occurring in the blood and nervous tissue of *Helix pomatia*. Comp. Gen. Pharmacol., 3, 171-174, (1972c).

OSBORNE, N.N. and COTTRELL, G.A. Transport of amines along the visceral nerve of *Helix pomatia*. Z. Zellforsch. Mikrosk. Anat., 109, 171-179, (1970).

OSBORNE, N.N. and COTTRELL, G.A. Distribution of biogenic amines in the slug, *Limax maximus*. Z. Zellforsch. Mikrosk. Anat., 112, 15-30, (1971).

OSBORNE, N.N. and COTTRELL, G.A. Amine and amino acid microanalysis of two identified snail neurons with known characteristics. Experientia. 28, 656-658, (1972a).

OSBORNE, N.N. and COTTRELL, G.A. The effect of optic tentacle removal on the transmitter content of the giant serotonin cell of *Helix aspersa*. J. Neurochem., 19, 2363-2368, (1972b).

OSBORNE, N.N., BRIEL, G. and NEUHOFF, V. Distribution of GABA and other amino acids in different tissues of the gastropod mollusc *Helix pomatia* including *in vitro* experiments with ^{14}C glucose and ^{14}C glutamic acid. Internat. J. Neurosci., 1, 265-272, (1971).

OSBORNE, N.N., POWELL, D. and COTTRELL, G.A. The effect of electrical stimulation on the levels of five amino acids and related compounds in the snail brain. Brain Research, 41, 379-386, (1972).

PARE, C.M.B. 5-Hydroxyindoles in phenylketonuric and nonphenylketonuric mental defectives. Adv. Pharmacol., 6B, 159-165, (1968).

PELLEGRINO DE IRALDI, A., GUEDET, R. and SUBURO, A.M. Differentiation between 5-hydroxytryptamine and catecholamines in synaptic vesicles. In: Prog. in Brain Res., 34, Ed. ERANKO, O. pp.161-170, (1971).

PERRY, W.L.M. Introductory remarks to indolealkylamines and behavior. In: Adv. Pharmacol., 6B, 209-211, (1968).

PETERS, D.A.V., McGEER, P.L. and McGEER, E.G. The distribution of tryptophan hydroxylase in cat brain. J. Neurochem., 15, 1431-1435, (1968).

PHILLIS, J.W. The pharmacology of synapses. (Int. Series Monographs in Pure and Applied Biology - 43), Pergamon Press, Oxford, (1970).

QUAY, W.B. Indole derivatives of pineal and related neural and retinal tissues. Pharmacol. Rev., 17, 321-345, (1965) .

QUAY, W.B. Comparative physiology of serotonin and melatonin. Adv. Pharmacol., 6A, 283-297, (1968).

RAPPORT, M.M., GREEN, A.A. and PAGE, I.H. Serum vasoconstrictor (serotonin). IV. Isolation and characterization. J. Biol. Chem., 176, 1243-1251, (1948).

ROBERTS, G.C.K. The formation of complexes between 5-hydroxytryptamine, adenosine triphosphate and bivalent cations in vitro. Biochem. J., 100, 30P, (1966).

ROBINSON, G.A., BUTCHER, R.W. and SUTHERLAND, E.W. "Cyclic AMP" Ann. Rev. Biochem., 37, 149-174, (1968).

ROGERS, D.C. Fine structure of the Epineural Connective Tissue Sheath of the Suboesophageal Ganglion in Helix aspersa. Z. Zellforsch., 102, 99-113, (1969).

ROSENBLUTH, J. The visceral ganglion of Aplysia californica. Z. Zellforsch., 60, 213-236, (1963a).

ROSENBLUTH, J. Fine structure of epineural muscle cells in Aplysia californica. J. Cell Biol., 17, 455-460, (1963).

ROSS, S.B. and RENYI, A.L. Accumulation of tritiated 5-hydroxytryptamine in brain slices. Life Sci., 6, 1407-1415, (1967).

RUDE, S., COGGESHALL, R.E. and VAN ORDEN, L.S. Chemical and ultrastructural identification of 5-hydroxytryptamine in an identified neuron. J. Cell Biol., 41, 832-854, (1969).

RUEGG, J.C. Actomyosin inactivation by thiourea and the nature of viscous tone in a molluscan smooth muscle. Proc. Roy. Soc. B, 158, 177-195, (1963).

SAKHAROV, D.A. Cellular aspects of invertebrate pharmacology. Ann. Rev. Pharmacology. ~~Ann. Rev. Pharmacol.~~, 10, 335-352, (1970).

SAKHAROV, D.A. and Zs-NAGY, I. Localization of biogenic monoamines in the cerebral ganglia of Lymnaea stagnalis. Acta. biol. Szeged., 19, 145-157, (1968).

SANCHIS, G.A. and ZAMBRANO, D. The structure of the central nervous system of a Pulmonate Mollusc (Cryptomphallus aspersa). I. Ultrastructure of the Connective Epineural Sheath. Z. Zellforsch., 94, 62-71, (1969).

SANDLER, M. The role of 5-hydroxyindoles in the carcinoid syndrome. Adv. Pharmacol., 6B, 127-142, (1968).

SATTELLE, D.B. and LANE, N.J. Architecture of gastropod central nervous tissues in relation to ionic movements. Tissue & Cell, 4, 253-270, (1972).

SCHLOTE, F.W. Submikroskoposche Morphologie von Gastropodennerven. Z. Zellforsch. 45, 543-568, (1957).

SCHMALZ, E. Zur Morphologie des Nervensystems von Helix pomatia L. Z. für wiss. Zool., 111, 506-568, (1914).

SCHMEKEL, L. and MECHSLER, W. Elektronmikroscopische Untersuchungen an Cerebro--Pleural-Ganglion von Nudibranchiern. I. Die Nervenzellen. Z. Zellforsch. Mikros. Anat., 89, 112-132, (1968).

SCHMIDT, G. Blutgefäßsystem und Mantelhöhle der Weinbergschnecke (Helix pomatia) Z. wiss. Zool. 115, 201-216, (1916).

SCHWARTZKOPFF, J. Über die Leistung des isolierten Herzens der Weinbergschnecke (H. pomatia) im künstlichen Kreislauf. Z. vergl. Physiol., 36, 543-594, (1954).

SEDDEN, C.B., KERAUT, G.A. and WALKER, R.J. The localisation of dopamine and 5-hydroxytryptamine in neurons of Helix aspersa. Symp. Zool. Soc. Lond., 22, 19-32, (1968).

SEGURA, E.T., BISCARDI, A.N. and APELBAUM, J. Seasonal variations of brain epinephrine, norepinephrine and 5-hydroxytryptamine associated with changes in the EEG of the toad, Bufo arenarium Hensel. Comp. Biochem. Physiol., 22, 843-850, (1967).

SHORE, P.A. Introductory remarks to the pharmacology of Indolealkylamines. In: Advances in Pharmacology, 6B, 3, (1968).

SMITH, T.A. The occurrence, metabolism and functions of amines in plants. Biol. Rev., 46, 201-241, (1971).

S.-RÓZSA, K. The influence of 5-HT on the heart phosphorylase activity in the snail, Lymnaea stagnalis. Life Sci., 8, (Part II), 229-234, (1969a).

S.-RÓZSA, K. Pharmacological investigations on the 5-hydroxytryptamine and noradrenaline receptors of gastropoda (Helix pomatia L.) heart. Annal. Biol. Tihany, 36, 57-62, (1969b).

S.-RÓZSA, K. and GRAUL, C. Is serotonin responsible for the stimulative effect of the extracardiac nerve in Helix pomatia? Ann. Biol. Tihany, 31,

S.-RÓZSA, K. and PECSI, T. Investigation of the role and mechanism of effect of nucleotides on the isolated hearts of molluscs. Annal. Biol. Tihany, 25, 61-74, (1968).

S.-RÓZSA, K. and PERENYI, L. Chemical identification of the excitatory substance released in Helix heart during electrical stimulation of the cardiac nerve. Comp. Biochem. Physiol., 19, 105-113, (1966).

S.-RÓZSA, K. and Zs-NAGY, I. Physiological and histochemical evidence for neuroendocrine regulation of heart activity in the snail Lymnaea stagnalis L. Comp. Biochem. Physiol., 23, 373-382, (1967).

STRETTON, A.O.W. and KRAVITZ, E.A. Neuronal geometry: determination with a technique of intracellular dye injection. Science, 162, 132-134, (1968).

SUTHERLAND, E.W. and ROBISON, G.A. The role of cyclic -3', 5'-AMP in responses to catecholamines and other hormones. Pharmac. Rev., 18, 145-161, (1966).

SUTHERLAND, E.W., RALL, T.W. and MENON, T. Adenyl cyclase. J. Biol. Chem., 237, 1220-1227, (1962).

SZCZEPANIAK, A.C. and COTTRELL, G.A. Biphasic action of glutamic acid and synaptic inhibition in an identified serotonin-containing neurone. Nature New Biol., 241, 62-63, (1973).

TAUC, L. Sur la nature de l'onde de surpolarisation de longue durée observée parfois après l'excitation synaptique de certaines cellules ganglionnaires des Mollusques. Comptes Rendus Séanc. Acad. Sci. Paris, 249, 318-320, (1959).

TAUC, L. and GERSCHENFELD, H.M. Cholinergic transmission mechanisms for both excitation and inhibition in molluscan central synapses. Nature, 192, 366-367, (1961).

TAXI, L. and GAUTRON, J. Données cytochimiques en faveur de l'existence de fibre nerveuses sérotoninergiques dans le coeur de l'Aplysie, Aplysia californica. J. Microscopic, 8, 627-636, (1969).

TEBĒCIS, A.K. and DI MARIA, A. A re-evaluation of the mode of action of 5-hydroxytryptamine on lateral geniculate neurones: comparisons with catecholamines and LSD. Exptl. Brain Research, 14, 480-493, (1972).

TSUDA, H., NOGUCHI, T. and KIDO, R. 5-Hydroxytryptophan pyrrolase in rat brain. J. Neurochem., 19, 887-890, (1972).

TWAROG, B.M. Responses of a Molluscan Smooth Muscle toACh and 5-HT. J. Cell Comp. Physiol., 44, 141-163, (1954).

TWAROG, B.M. Effects of acetylcholine and 5-Hydroxytryptamine on the contractio of a molluscan smooth muscle. J. Physiol., 152, 236-242, (1960).

TWAROG, B.M. The regulation of catch in molluscan muscle. J. Gen. Physiol., 50, 157-169, (1967a).

TWAROG, B.M. Factors influencing contraction and catch in Mytilus smooth muscle. J. Physiol. (Lond.), 192, 847-856, (1967b).

TWAROG, B.M. Possible mechanism of action of serotonin on Molluscan muscle. Adv. Pharmacol., 6B, 7-15, (1968).

TWAROG, B.M. and HIDAKA, T. The calcium spike in Mytilus muscle and the action of serotonin. J. Gen. Physiol., 57, 252-254, (1971).

TWAROG, B.M., COTTRELL, G.A. and MUNEOKA, Y. Effects of changes in the ionic environment of the action of serotonin on Mytilus smooth muscle. J. Gen Physiol., 58, 706-710, (1971).

UVSPÄÄ, V.J. The 5-hydroxytryptamine content of the brain and some other organs of the hedgehog (Erinaceus europaeus) during activity and hibernation. Experientia, 19, 156-158, (1963).

WAY, E.L. Role of serotonin in morphine effects. Fed. Proc., 31, 113-120, (1972).

WEBER, L.J. and HORITA, A. A study of 5-hydroxytryptamine formation from L--tryptophan in the brain and other tissues. Biochem. Pharmacol., 14, 1141-1149, (1965).

WEINREICH, D., DEWHURST, S.A. and McCAMAN, R.E. Metabolism of putative transmitters in individual neurons of Aplysia californica: aromatic amino acid decarboxylase. J. Neurochem., 19, 1125-1131, (1972).

WEISS, B.L. and AGHAJANIAN, G.K. Activiation of brain serotonin metabolism by heart: role of midbrain raphe neurons. Brain Res., 26, 37-48, (1971).

WEISS, G.B. and ROSECRANS, J.A. Analysis of 5-hydroxytryptamine -^{14}C uptake and metabolism in intestinal smooth muscle. Europ. J. Pharmacol., 13, 197-207, (1971).

WELSH, J.H. Distribution of serotonin in the nervous system of various animal species. Adv. Pharmacol., 6A, 171-186, (1968).

WELSH, J.H. Neurobehavioral regulation and the pharmacology of a molluscan heart. Comp. Gen. Pharmacol., 2, 423-432, (1971).

WELSH, J.H. and MOORHEAD, M. The in vivo synthesis of 5-hydroxytryptamine from 5-hydroxytryptophan by nervous tissues of two species of molluscs. GUMNA J. MED. Sci. (Japan), 8, 211-216, (1959).

WELSH, J.H. and MOORHEAD, M. The quantitative distribution of 5-hydroxytryptamine in the invertebrates, especially in the nervous system. J. Neurochem., 6, 146-169, (1960).

WERMAN, R. Criteria for identification of a central nervous system transmitter. Comp. Biochem. Physiol., 18, 745-766, (1966).

WHITTAKER, V.P. et al. The separation of synaptic vesicles from nerve-ending particles ('Synaptosomes'). Biochem. J. 90, p.293-303, (1964).

WHITTAKER, V.P. Subcellular localization of Neurotransmitters. In: Advances in Cytopharmacology. Ed. CLEMENTI, F. and CECCARELLI, B. Raven Press, New York, pp.319-330, (1971).

WIERSMA, C.A.G. The organization of the arthropod nervous system. Amer. Zool., 2, 67-78, (1962).

WILLOWS, A.O.D. and HOYLE, G. Neuronal network triggering a fixed action pattern. Science, 166, 1549-1551, (1969).

WILSON, O.M. and LARIMER, J.L. The catch property of ordinary muscle. Proc. Nat. Acad. Sci. U.S.A., 61, 909-916, (1968).

WOOD, J.G. Electron microscopic localization of 5-hydroxytryptamine (5-HT). Texas Rep. Biol. Med., 23, 828-837, (1965).

WOOD, J.G. Electron microscopic localization of amines in central nervous tissue. Nature, 209, 1131-1133, (1966).

WURTMAN, R.J. and FERNSTROM, J.D. L-tryptophan, L-tyrosine, and the control of brain monoamine synthesis. In: Perspectives in Neuropharmacology. Ed. SNYDER, S.H., pp.143-193, Oxford Univ. Press, (1972).

YORK, B. and TWAROG, B.M. Evidence for the release of serotonin by relaxing nerves in molluscan muscle. Comp. Biochem. Physiol., 44A, 423-430, (1973).

ZIEHER, L.M. and DE ROBERTIS, E. Subcellular localization of 5-hydroxytryptamine in rat brain. Biochem. Pharmacol., 12, 596-598, (1963).

Zs-NAGY, I. Histochemical demonstration of biogenic amines in the central nervous system of the lamellibranch mollusc Anodonta cygnea L. Acta biol. Szeged., 18, 1-8, (1967).

Zs-NAGY, I. and BOROVYAGIN, V.L. Organisation of the cytosomal membranes of molluscan neurons under normal and anaerobic conditions as revealed by electron microscopy. Tissue and Cell, 4, 73-84, (1972).

Zs-NAGY, I. and SAKHAROV, D.A. Axosomatic synapses in procerebrum of Gastropoda. Experientia, 25, 258-259, (1969).

Zs-NAGY, I. and SAKHAROV, D.A. The fine structure of the procerebrum of pulmonate molluscs, Helix and Limax. Tissue and Cell, 2, 399-411, (1970).

Zs-NAGY, I. and S.-RÓZSA, K. The ultrastructure and histochemical properties of the granulated cells in the heart of the snail Lymnaea stagnalis L. Acta biol. Acad. Sci. Hung., 21, 121-133, (1970).

Zs-NAGY, I., ROSZA, K.S., SALANKI, J., FÜLDES, J., PERENYI, I. and DEMETER, M. Subcellular localization of 5-hydroxytryptamine in the central nervous tissue of lamellibranchiates. J. Neurochem., 12, 245-251, (1965).

SUMMARY

An outline of the present knowledge of neuronal 5-HT in molluscs is given. The purpose of the present study was to obtain information on the structure of 5-HT-containing neurons, on the mechanisms of transport of 5-HT and its precursors into and within neurons, on the nature of the blood supply to the CNS, and on the function of 5-HT-containing neurons within the CNS of Helix pomatia. The following observations were made:

1. There is a giant serotonin-containing neuron (GSC) in each cerebral ganglion of Helix pomatia. Presynaptic endings of the GSCs are located in the buccal ganglia and peripheral musculature. Dense-cored vesicles of mean diameter 100 nm were observed in the perikarya and the axon branches of the GSCs within the cerebral ganglia. Evidence is presented which suggests that these vesicles sequester 5-HT. Vesicles with a similar appearance are present in the presumed synaptic endings of the GSC. Axonal processes containing agranular vesicles (mean diameter 50 nm), or granular vesicles (50 - 100 nm diameter), or both, make close contact with the fine axon branches of the GSCs within the cerebral ganglion neuropile. Some of these axon processes appear to be presynaptic endings making synaptic connections with the GSCs.

2. Each paired ganglion of H. pomatia is supplied by symmetrically arranged branches from the anterior aorta. Capillaries from these branches open into a blood space which is adjacent to, and continuous over the surface of the nervous tissue. Blood passes from this space through the epineural sheath into the body cavity sinuses. Three tissue layers separate the blood spaces from the neurons of each ganglion. These are (i) a luminal endothelium, (ii) a fibrous connective tissue layer which is mainly collagen, and (iii) glial cells. Both the luminal endothelium and connective tissue are freely permeable to uncharged particles of 10 nm or less.

3. Following exposure to tritiated 5-HT, electron microscope autoradiography showed that silver grains, often in very high concentrations, were located only over certain fine axon branches and processes thought to be nerve endings.

These processes contained small dense-cored vesicles, morphologically identical to those thought to sequester 5-HT in the perikarya of the GSCs. It is suggested that re-uptake is a mechanism of inactivation of 5-HT in the CNS of Helix pomatia.

4. Following exposure to tritiated 5-HTP, silver grains were observed over the perikarya of the GSCs and other known 5-HT-containing neurons in light and electron microscope autoradiograms. There was no indication that the 5-HTP was taken up by nerve endings or by non-nervous structures. The accumulation of tritiated tryptophan was less specific; all the neuron perikarya showed an accumulation of radioactivity after exposure to this substance.

5. Electrophysiological analysis showed that each GSC sends axon branches to muscles in the lips of the animal. Selective stimulation of the GSC resulted in an increase of electrical activity recorded from these muscles, but no change in their length. This effect was mimicked by 5-HT applied to the muscles. It is suggested that the GSC has a facilitatory effect on the lip muscle potentials.

The advantages of using large molluscan neurons for studying neuronal 5-HT are discussed. It is concluded that the 5-HT within the GSCs of H. pomatia serves a neurotransmitter role.

PUBLICATIONS

Parts of this thesis have been published, or are in the press:

Chapter 1. PENTREATH, V.W., OSBORNE, N.N. and COTTRELL, G.A. 1973. Anatomy of giant serotonin-containing neurones in the cerebral ganglia of Helix pomatia and Limax maximus. Z. Zellforsch. In press.

Chapter 2. PENTREATH, V.W. and COTTRELL, G.A. 1970. The blood supply to the central nervous system of Helix pomatia. Z. Zellforsch., 111 , 160-178.

Chapter 3. PENTREATH, V.W. and COTTRELL, G.A. 1972. Selective uptake of 5-hydroxytryptamine by axonal processes in Helix pomatia. Nature (New Biol.), 239, 213-214.

Chapters 3,4. PENTREATH, V.W. and COTTRELL, G.A. 1973. Uptake of serotonin, 5-hydroxytryptophan and tryptophan by giant serotonin-containing neurones and other neurones in the central nervous system of the snail (Helix pomatia). Z. Zellforsch. In press.

Chapter 5. PENTREATH, V.W. 1973. Effect of stimulating a central giant serotonin-containing neuron on peripheral muscles in the snail Helix pomatia. Experientia. In press.